Effects of serotonin signaling on behavior and gene transcription

A journey through postnatal brain development

Yvet Kroeze

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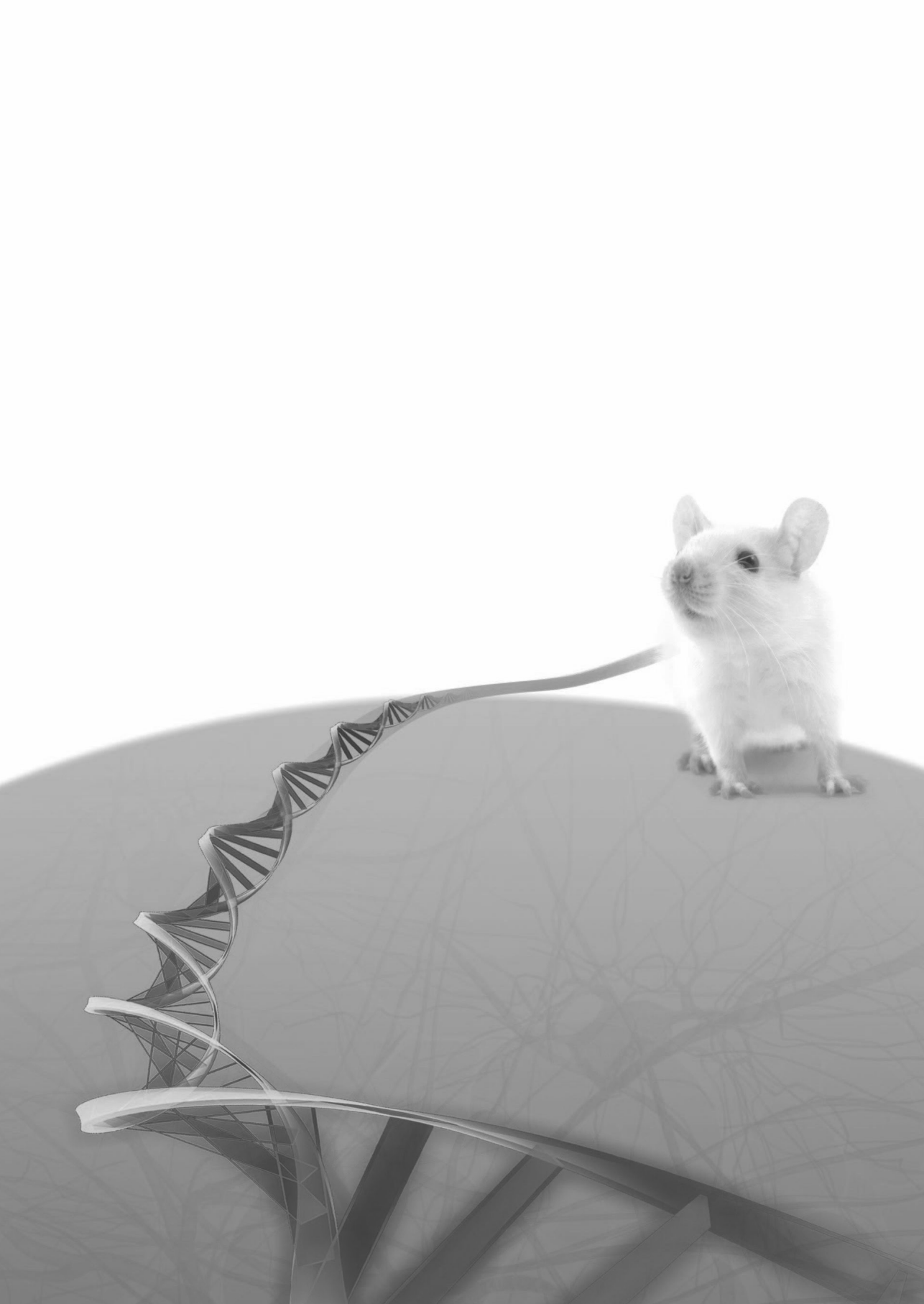

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General introduction and outline of the thesis

Affective disorders

Affective disorders are a set of psychiatric diseases which typically affect mood. The main types of affective disorders are major depressive disorder (further called depression in this chapter), anxiety-related disorders and bipolar disorder. Affective disorders are among the leading causes of burden worldwide. Depression and anxiety-related disorders were, respectively, the second and sixth leading cause of years lived with disability in 2010[1,2]. The 12 month prevalence of depression, bipolar disorder and anxiety-related disorders are respectively, 6.9 percent, 2.6 percent and 18 percent of all U.S. adults[3,4]. Pregnancy is a period with high risk for depression, particularly for women with pre-existing psychiatric disorders. The number of women that are depressed during pregnancy ranges between 7% and 26%[5-8]. Depression is often caused by experiencing unavoidable stress, while anxiety is caused by stressors which are avoidable and which are not present at that moment. The symptoms for each affective disorder can vary greatly, but there are some common signs. Depression is characterized by prolonged sadness, anxiety, lack of energy, lack of interest in normal activities (anhedonia), suicidal thoughts and major changes in eating and sleeping habits. Bipolar disorder is defined by unusual and chronic mood swings with during the depressive phase symptoms similar to those during depression and during the (hypo)manic phase people feel extremely positive and active with symptoms like, less sleep and feelings of exaggerated self-confidence, irritability, aggression, self-importance and impulsiveness. There are different types of anxiety-related disorders, which are all characterized by feelings of nervousness, anxiety and fear. All these symptoms can have a strong impact on people's daily life. The Diagnostic and Statistical Manual of Mental Disorders, fifth revision (DSM-5)[9] and the International Classification of Diseases[10] are the current standards by which affective disorders are classified and which provides the language by which clinicians, researchers, and public health officials communicate about mental disorders. Patients can be diagnosed for a specific affective disorder while displaying variable sets of symptoms, see Box 1. As a consequence, patients can vary considerably in clinical presentation. Besides the symptom-based diagnosis of affective disorders there are also studies focusing on discovering new markers for the diagnosis of affective disorders, such as endophenotypes[11,12].

Box 1: DSM-5 Criteria

Major Depressive episode

A. Five or more of the following symptoms have been present and documented during the same two-week period and represent a change from previous functioning; at least one of the symptoms is either (1) depressed mood or (2) loss of interest or pleasure:
   1. Depressed mood most of the day, nearly every day
   2. Markedly diminished interest or pleasure in all, or almost all, activities most of the day, nearly every day
   3. Significant weight loss when not dieting or weight gain, or decrease or increase in appetite nearly every day

4. Insomnia or hypersomnia nearly every day
5. Psychomotor agitation or retardation nearly every day
6. Fatigue or loss of energy nearly every day
7. Feelings of worthlessness or excessive or inappropriate guilt nearly every day
8. Diminished ability to think or concentrate, or indecisiveness, nearly every day
9. Recurrent thoughts of death (not just fear of dying), recurrent suicidal ideation without a specific plan, or a suicide attempt or a specific plan for committing suicide

B. The symptoms cause clinically significant distress or impairment in social, occupational, or other important areas of functioning

C. The episode is not attributable to the physiological effects of a substance or to another medical condition.

Generalized Anxiety Disorder

A. Excessive anxiety and worry, occurring more days than not for at least 6 months, about a number of events or activities

B. The individual finds it difficult to control the worry

C. The anxiety and worry are associated with three (or more) of the following six symptoms (with at least
some symptoms having been present for more days than not for the past 6 months):
    1. Restlessness, feeling keyed up or on edge
    2. Being easily fatigued
    3. Difficulty concentrating or mind going blank
    4. Irritability
    5. Muscle tension
    6. Sleep disturbance (difficulty falling or staying asleep, or restless, unsatisfying sleep)

D. The anxiety, worry, or physical symptoms cause clinically significant distress or impairment in social, occupational, or other important areas of functioning

E. The disturbance is not attributable to the physiological effects of a substance or another medical condition

F. The disturbance is not better explained by another medical disorder

Brain regions involved in affective disorders

Years ago, a couple of brain structures were suggested to be responsible for emotion and emotional expression. This became known as the 'limbic system,' based on the 'grand lobe limbique' of Broca[13]. Limbic refers to an edge or border and in this case indicates the structures around the medial edge of the cerebral hemisphere. There is no universal agreement on the total list of structures which comprise the limbic system, but the best known limbic structures are the hypothalamus, the amygdala and the hippocampus. In addition, the prefrontal cortex (PFC) is a cortical region which has intensive neural connections with limbic structures and is implicated in emotion and affective disorders. I will briefly discuss the functions of these

brain regions and their relation with affective disorders, with the main focus on the PFC and the hippocampus, since these structures are used for molecular experiments in this thesis.

The amygdala has a primary role in emotional reactions and also in the processing of memory and decision-making[14,15]. The cortical projections of the amygdala are widespread, but the strongest connections are with the medial PFC (mPFC)[16]. The hypothalamus contains a number of small nuclei with a variety of functions, amongst others, controlling body temperature, hunger, fatigue, sleep, circadian rhythms and response to stress. The hypothalamus links the nervous system to the endocrine system via the pituitary gland (hypophysis). With regard to stress, the paraventricular nucleus of the hypothalamus secretes corticotropin-releasing hormone, which is involved in a stress response via the hypothalamic–pituitary–adrenal axis (HPA axis), leading to the release of corticosterone[17,18]. The hippocampus plays important roles in spatial navigation, learning and the consolidation of information for long- and short-term memory. Although learning and memory is the main role of the hippocampus, it is strongly connected to parts of the brain that are involved in emotional behavior, like the amygdala[19]. The glutamatergic pyramidal and granule cells represent 90% of hippocampal neurons and the remaining 10% are primarily γ-aminobutyric acid (GABA)-producing interneurons[19,20]. In addition, receptors from other neurotransmitters, such as serotonin[21-23], are also present in the hippocampus. The laminae that make up the hippocampus consist of the dentate gyrus (DG), the cornu Ammonis (CA) and the hilus (also called CA4). The cornu Ammonis can be divided into three regions, termed CA1–CA3, based on pyramidal neuron morphology and sensitivity to anoxia[19]. The DG consists of three layers of neurons; molecular, granular and polymorphic layer. The granular layer is most prominent and contains granule cells that project to the CA3 region[24]. The DG is one of the two brain regions where adult neurogenesis takes place. In patients with depression a smaller hippocampal volume has been observed[25]. Volume reductions of the hippocampus might be the result of remodeling of key cellular elements, involving retraction of dendrites, decreased neurogenesis in the DG and loss of glial cells[26-30]. Adult neurogenesis in the DG has been shown to be reduced in depressed patients and can be increased by antidepressant treatment[31]. The PFC, as a conglomerate of subregions, is implicated in a plethora of cognitive functions, such as working memory, attention, arousal, decision making, moderating social behavior, behavioral inhibition, and motivation[32]. Each subregion of the PFC has its own specific role in cognition. The dorsolateral PFC (DLPFC) is involved in working memory; the ventrolateral PFC (VLPFC) plays a role in decision making and goal-directed behavior; the orbitofrontal cortex (OFC) signals the valence of predictive stimuli, and the mPFC exerts control over emotion and motivation[32-35]. The PFC is affected in many, if not all, neuropsychiatric disorders. For example, in several subregions of the PFC a reduction in gray matter volume has been observed in depression and bipolar disorder, with the most prominent volumetric abnormality reported in the anterior cingulate cortex (ACC) of the mPFC[36]. Furthermore, decreased mPFC neuronal activity is associated with depression- and anxiety-like behaviors in mice[37]. Recent evidence indicates that the mPFC is critical for the extinction of fear. Moreover, a dorsal-ventral

distinction is apparent within the mPFC, such that the rodent prelimbic cortex drives the expression of fear, whereas the rodent infralimbic cortex suppresses these behaviors after extinction. Posttraumatic stress disorder (PTSD) patients exhibit disturbed extinction of fear and a decreased activity within the ventral mPFC is observed in these patients when exposed to traumatic reminders[38-40].

The serotonergic system

Aberrant serotonin (5-hydroxytryptamine, 5-HT) signaling in the brain is associated with affective disorders[41-43]. Decades ago, studies showed that low extracellular 5-HT levels in the brain and a diminished activity of the serotonergic pathway play a causal role in the pathophysiology of depression[44]. Anxiety-related disorders and bipolar disorder are also associated with decreased serotonergic signaling[45,46]. 5-HT is a neurotransmitter primarily found in the gastrointestinal tract, blood platelets, and the central nervous system (CNS). Production of 5-HT takes place in the brain and the intestines. 5-HT can be found widely across the body and can influence a variety of body and psychological functions. 5-HT cannot cross the blood-brain barrier; therefore 5-HT that is used in the brain must be produced there. Brain-derived 5-HT plays a role in several psychological functions like mood, sleep, sexual activity, emotions and appetite. In the brain 5-HT is produced by the serotonergic neurons located in the raphe nuclei in the brainstem, which project to nearly all brain regions[47]. The majority of projections arise from the dorsal and median raphe nuclei[48], which innervate the amygdala, basal forebrain, hypothalamus, thalamus, caudate-putamen, cerebral cortex, and part of the hippocampus[47]. Most (95%) of the 5-HT in the body is located in the gut[49]. The gut is surrounded by enterochromaffin cells, which release 5-HT in response to food in the lumen, resulting in contraction around the food. Besides peristalsis, 5-HT can mediate vasoconstriction and perception of pain or nausea through activation of a diverse family of 5-HT receptors in the gastrointestinal tract[50]. Recent studies also suggest that gut-derived 5-HT influences bone metabolism[51]. In the blood, 5-HT is derived from enterochromaffin cells of the intestine, but its plasma concentration is primarily regulated by circulating platelets[52]. 5-HT acts as a vasoconstrictor to stop bleeding when platelets bind to damaged tissue and release 5-HT upon activation[53].

5-HT belongs to the group of classical monoamines. Classical monoamines consist of histamine, catecholamines (epinephrine, norepinephrine, dopamine) and tryptamines (serotonin, melatonin), which all contain one amino group that is connected to an aromatic ring by a two-carbon chain (-CH2-CH2-). 5-HT is synthesized from tryptophan (Figure 1). 5-HT is taken up into storage vesicles and can be released from these vesicles into the extracellular space, where it can activate subtypes of the 5-HT receptor. At present, 16 different 5-HT receptor subtypes have been identified: $5\text{-HT}_{1A,\ 1B,\ 1C,\ 1D,\ 1E,\ 1F}$, $5\text{-HT}_{2A,\ 2B,\ 2C}$, $5\text{-HT}_{3A,\ 3B}$, 5-HT_{4}, $5\text{-HT}_{5A,\ 5B,}$ 5-HT_{6} and 5-HT_{7}. Each receptor can activate its own signal transduction pathway inside the postsynaptic neuron. The 5-HT_{3A} and $_{3B}$ receptors are ligand-gated ion channel receptors, while all others are G-protein coupled receptors. Each 5-HT receptor is associated

with different functions, as shown in Table 1, which is a consequence of the different locations and downstream pathways of each receptor. The 5-HT transporter (5-hydroxytryptamine transporter, 5-HTT) facilitates the reuptake of 5-HT from the synaptic cleft back into the presynaptic neuron. Within the presynaptic neuron, 5-HT would either be taken up by the storage vesicles or degraded by monoamine oxidase A (MAO-A) (Figure 1).

Serotonin-related gene polymorphisms in affective disorders

Genetic variations, such as mutations and polymorphisms, contribute to the etiology of affective disorders. Bipolar disorder is highly heritable with genetic influences explaining 60–85% of the risk[76], while depression and anxiety-related disorders have a considerably lower heritability of, respectively, 37%[77] and 30-40%[78]. A polymorphism is a natural variation in a DNA sequence which occurs with fairly high frequency in the general population. Polymorphisms in genes coding for components of the serotonergic pathway are extensively studied in relation to affective disorders. A well-studied polymorphism is the 5-HTT-linked polymorphic region (5-HTTLPR). 5-HTTLPR is located in the promoter region of 5-HTT (*SLC6A4* gene) about 1kb upstream of the transcription start site[79]. The long (L) allele contains 16 repeats of a 20-23 bp repetitive sequence, whereas the short (S) allele contains 14 repeats. The deletion of 2 repeats is located 1212-1255 bp upstream of the transcription start site[80,81]. The S-allele is very common in the Caucasian population, with an allele frequency of around 40%. Frequencies differ between ethnicities, with lower S-allele frequencies in African-Americans (20-25%[82]) and higher frequencies in Asian populations (75-80%[83,84]). The S-allele is associated with reduced transcription of the *SLC6A4* gene. The S/S and the S/L genotypes both show an approximately two-fold lower transcriptional efficiency compared to the L/L genotype, which implies that the S-allele is dominant over the L-allele regarding expression of the *SLC6A4* gene.[81,85]. S-allele carriers have an increased risk of developing anxiety, depression and aggression[85-88]. The higher expression of the L-allele may be due to an A/G single nucleotide polymorphism (SNP) within the 5-HTTLPR of the L-allele, rs25531 (g.28564346A>G, dbSNP allele frequencies: A: 86%, G: 14%, all genomic locations are based on GRCh37.p13), resulting in the La (L-like) and Lg (S-like) allelic variants[89]. The Lg allelic carriers have 5-HTT levels more similar to S-allele carriers and are associated with greater depression severity compared with the higher expressing La-allele[90]. In addition to 5-HTT, SNPs in 5-HT receptors are also associated with affective disorders. The *HTR1A* gene, encoding for the 5-HT_{1A} receptor, contains a SNP, rs6295 (g.63258565G>C), which is related to the pathophysiology of affective disorders. The G variant of the rs6295 polymorphism in the promoter region of *HTR1A* has been associated with higher expression of 5-HT_{1A} receptors, depression, completed suicide and less stressors preceding the need of hospitalization for bipolar disorder[91,92]. Genotype frequencies of the *HTR2A* polymorphism rs6313 (g.47469940C>T) are associated with suicidal behavior (T-allele) and schizophrenia (CC genotype)[93,94]. The C-allele is associated with lower mRNA and lower protein expression[93]. Tryptophan hydroxylase 2 (TPH2) is considered as a rate-limiting enzyme in 5-HT biosynthesis. It catalyses the conversion of tryptophan to 5-hydroxytryptophan (Figure

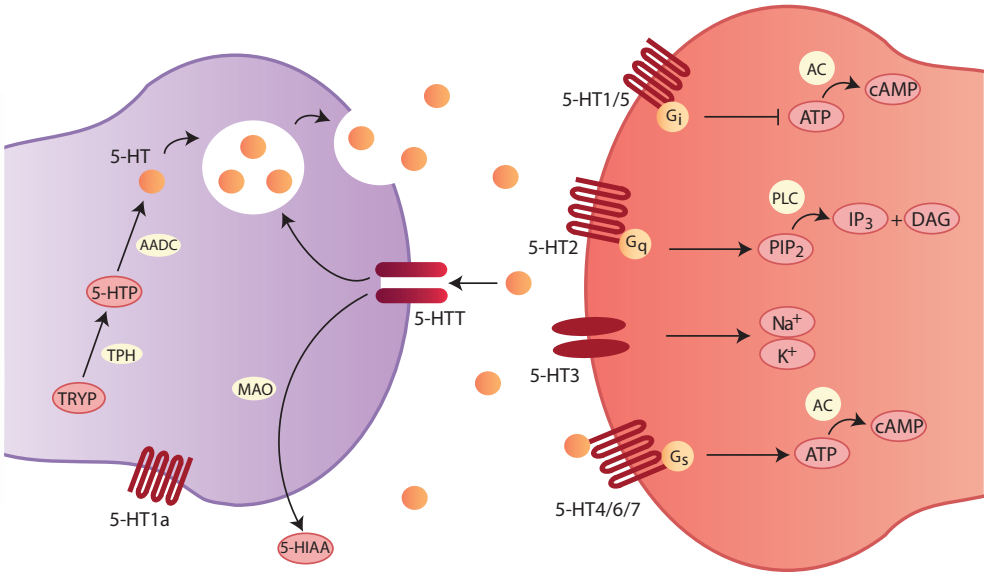

Figure 1. Schematic overview of the serotonergic system in the central nervous system. Serotonin (5-hydroxytryptamine, 5-HT) is synthesized by the conversion of tryptophan (TRYP) to 5-hydroxytryptophan (5-HTP) catalyzed by tryptophan hydroxylase (TPH), followed by conversion of 5-HTP to 5-HT catalyzed by aromatic amino acid decarboxylase (AADC). 5-HT is taken up into storage vesicles and can be released from these vesicles into the synaptic space, where it can activate subtypes of the 5-HT receptor (1–7). Each can activate its own signal transduction pathway inside the postsynaptic neuron. 5-HT is taken up into the presynaptic neuron by the 5-HT transporter (5-HTT). Within the presynaptic neuron, 5-HT is taken up by the storage vesicles or degraded by monoamine oxidase (MAO). 5-HIAA, 5 hydroxyindolacetic acid; AC, adenylate cyclase; ATP, Adenosine triphosphate; cAMP, Cyclic adenosine monophosphate; PLC, Phospholipase C; DAG, diacylglycerol; IP3, inositol-1,4,5-trisphosphate; PIP2, phosphatidylinositol-4,5-bisphosphate.

Table 1. Biological processes and disorders associated with 5-HT receptors

Receptor	Receptor function and associated dysfunctional phenotypes
5-HT_{1A}	Sleep[54], Pain[55], Anxiety[56], Depression[56], Aggression[57]
5-HT_{1B}	Vasoconstriction[58], Bone mass[59], Aggression[57]
5-HT_{1D}	Vasoconstriction[58]
5-HT_{2A}	Urinary bladder contractions[60], Depression[61], Anxiety[43], Schizophrenia[62]
5-HT_{2B}	Cardiovascular functioning[63,64]
5-HT_{2C}	Appetite[65], Bone mass[66], Depression[67], Schizophrenia[68]
5-HT_{3}	Emesis[69]
5-HT_{4}	Movement of food[70], Learning & memory[71], Depression[71]
5-HT_{5A}	Memory consolidation[72]
5-HT_{6}	Cognition[73], Depression[42], Anxiety[42]
5-HT_{7}	Sleep[74], Circadian rhythm[74], Thermoregulation[74], Vasodilation[75], Locomotion, Pain[75], Depression[74,75]

Note: Not for all receptors a function is known (yet), therefore several receptors are missing in this table

1). The A-allele of the *TPH2* SNP rs120074175 (g.72425324G>A) showed an association with depression[95]. TPH1, sharing 70% homology in amino acid sequence with TPH2, is expressed in the gastrointestinal tract and pineal gland and is an important factor in the 5-HT synthesis[96]. It was found that the C-allele of the rs2108977 SNP (g.18040596T>C) of *TPH1* conferred a protective effect against the development of PTSD[97] and depression[98]. The rs1137070 SNP (g.43603391T>C) in *MAOA*, encoding for the mitochondrial enzyme catalyzing oxidative deamination of 5-HT, is associated with depression. The T-allele appeared with a significantly higher frequency in depressed female patients than in the control group[99]. However there are also several studies showing no association between SNPs in serotonergic genes and affective disorders[100-103].

Gene-environment interactions in affective disorders

It is becoming increasingly evident that the vulnerability to affective disorders is dependent on the interplay of the genome and the environment, also called gene-environment interactions. Environmental factors like stress, abuse, diet and trauma contribute to the etiology of affective disorders. Some people are more resilient to a certain environmental trigger than others, which can (at least partly) be explained by their genetic background. Genetic variants, such as the SNPs mentioned in the previous paragraph, confer differential susceptibility to a particular environmental factor. A mechanism by which environmental factors can influence gene expression is epigenetics. Epigenetics refers to modifications that occur on top of genetics *(epi* means *on top of*), which are modifications that influence gene expression without changing the DNA code. Examples of epigenetic modifications are DNA methylation and histone modifications. In addition, non-coding RNAs (ncRNAs) can influence gene expression (Figure 2). DNA methylation, a relatively stable mark, mainly occurs at cytosines present in cytosine-phosphate-guanine (CpG) sites and is catalyzed by DNA methyltransferases (DNMTs)[104]. In mammals, most patterns of DNA methylation are established during embryonic development by DNMT3A and DNMT3B and maintained by a DNMT1-mediated copying mechanism when cells divide[105]. DNA methylation of cytosines (5-methylcytosine, 5mC) is generally associated with repression of gene expression[104]. Aberrant DNA methylation patterns in the brain are linked to affective disorders[106,107]. Genome-wide twin studies showed aberrant DNA methylation in peripheral blood of depressed patients[107]. In addition, bipolar disorder is associated with aberrant DNA methylation observed in blood and brain tissue using several gene-specific and genome-wide approaches[108]. Anxiety has been shown to be associated with higher global levels of DNA methylation in the blood[109]. Also increased gene-specific DNA methylation was observed in saliva of older women with anxiety and depression[110]. Whether these DNA methylation changes are cause or consequence of the phenotype is unknown. 5-hydroxymethylcytosine (5hmC), created by oxidation of 5-methylcytosine (5mC) by TET proteins, is thought to be an intermediate step in the active demethylation process. However, there is increasing evidence that 5hmC is not simply an intermediate step, but also exerts its own regulatory functions such

as promoting genome stability, marking sites of DNA damage, regulation of gene expression and tissue differentiation[111,112]. 5hmC is highly abundant in the brain[113]. There are a few recent studies showing a potential link between 5hmC and early-life stress[114] and early-life stress-induced anxiety[115]. Furthermore, lower 5hmC levels were observed in blood samples of bipolar disorder patients[116]. Histone modifications are more reversible epigenetic marks[104] and comprise acetylation, methylation, phosphorylation and ubiquitination[104]. The type of modification and the position on the histone protein determine whether the modification leads to a more open or a more condensed chromatin structure, which is associated with transcriptional activation or repression, respectively[117]. Especially histone acetylation has been shown to correlate with affective disorders[118]. Several studies showed increases in histone deacetylase 5 (HDAC5) levels in the blood of depressed patients[119,120], while brain levels of HDAC5 are shown to be reduced in depressed patients[121,122]. Increased histone H3 acetylation and decreased levels of HDAC2 in the nucleus accumbens have been observed in postmortem brain tissue from depressed patients[122]. ncRNAs have emerged as key regulators of protein-coding gene expression. ncRNAs can be short (< 200 nucleotides) or long (< 200 nucleotides) and are further subdivided based on their genomic origin and mechanism of action[123]. Small ncRNAs can regulate transcription by guiding Argonaute protein complexes to complementary nascent RNA scaffolds and then mediating the recruitment of histone and DNA methyltransferases[124]. Long ncRNAs can regulate transcription by recruiting polycomb proteins, which remodel chromatin[125] and by acting as enhancers[123,126,127]. Several long and

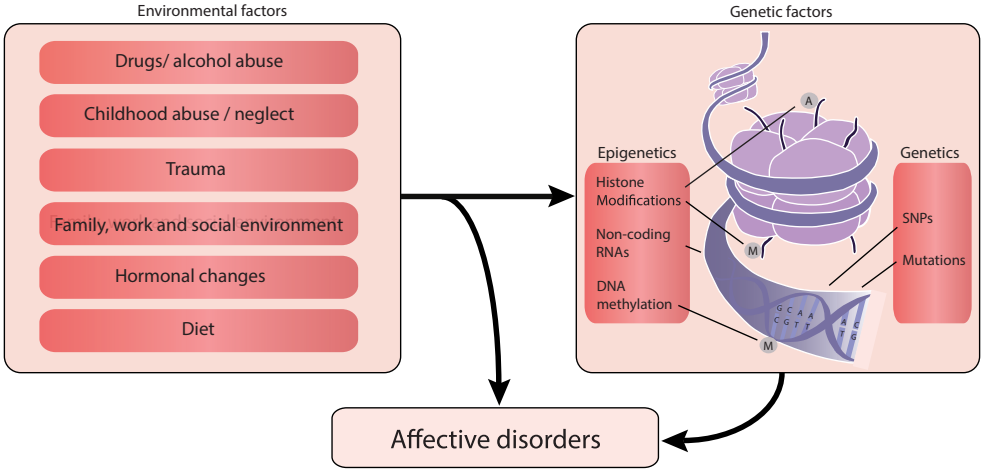

Figure 2. Environmental and genetic factors associated with affective disorders. Environmental factors influencing affective disorders are drugs/alcohol abuse, childhood abuse/neglect, trauma, hormonal changes (mostly postpuberal), diet, family and conditions at home, school, office including a wide array of factors such as socio-economic status, IQ, education, etc. (Epi)genetic factors include mutations (insertions and deletions of nucleotides causing frame shifts and substitutions), Single nucleotide polymorphisms (SNPs) and epigenetic regulation by non-coding RNAs, DNA (hydroxy)methylation, histone modifications (acetylation, phosphorylation, methylation etc. of histone tails protruding from the nucleosome). M: methylation, A: acetylation

short ncRNAs have been implicated in neurodevelopment, brain function and neurological diseases[128-130], however the role of ncRNAs in affective disorders is largely unknown. The most studied ncRNAs are microRNAs (miRNA, a short ncRNAs) and a few are found to be associated with bipolar disorder[131], anxiety-related disorders[132] and depression[133].

Epigenetic regulation of several serotonergic genes has been associated with affective disorders. Both increased and decreased DNA methylation of the *SLC6A4* (encoding for 5-HTT) promoter have been associated with depression[106]. An increase in *TPH2* promoter methylation was associated with depression and suicide[134]. *Htr1a* (encoding for 5-HT_{1A}) promoter methylation was increased in mice subjected to unpredictable chronic mild stress to induce a depression-like phenotype[135]. Furthermore, aberrant DNA methylation of *SLC6A4*, *HTR1A* and *HTR2A* is associated with bipolar disorder[108]. While several studies investigate DNA methylation, there are hardly any studies investigating the effect of histone modifications of serotonergic genes in affective disorders. There is one study showing increased acetylation of histone H3 at the promoter of the *MAOA* gene in rats exposed to peripuberty stress[136]. Also some serotonin-related non-coding RNAs are linked to affective disorders. MiR-16, which targets 5-HTT, was significantly lower in cerebrospinal fluid (CSF) from depressed patients compared to controls. Another 5-HTT associated miRNA, miR-135, was found to control the onset of co-existing depression and anxiety symptoms in mice[137,138] as well as the response to antidepressant treatment[137,138]. In addition, miR-96 targets 5-HT_{1B} and is associated with aggression-related disorders[139]. The discovered (epi)genetic contributors and their pathways can be used in the treatment of affective disorders.

Treatment of affective disorders

Treatment of affective disorders consists of drug administration and therapy. Drugs used for treatment of depression and anxiety-related disorders are selective serotonin reuptake inhibitors (SSRIs), serotonin-norepinephrine reuptake inhibitors (SNRIs), norepinephrine reuptake inhibitors (NRIs), tricyclic antidepressants (mostly replaced by other drugs due to unpleasant side effects) and monoamine oxidase inhibitors (MAOIs). All drugs act by increasing the availability of (extracellular) monoamines, either by blocking the reuptake in the presynaptic neuron or by inhibition of 5-HT degradation. Recently several clinical trials with new drugs acting on the glutamate system (mostly acting on N-methyl-D-aspartate (NMDA) receptors) are conducted in depressed patients and their results are promising[140]. The treatment of depression in people with bipolar disorder is similar to that for people who develop depression without episodes of mania. In addition, the (hypo)manic period in bipolar disorder is treated with mood stabilizers[141], antipsychotics[141] and sedatives[142]. Besides medication, different therapies are used for the treatment of affective disorders such as psychotherapy[143], exposure therapy[144] and electro-convulsive therapy[145].

Among the most frequently prescribed drugs for affective disorders are SSRIs. SSRIs are best known as antidepressants, but they are also used in the treatment of anxiety-related disorders, like obsessive compulsive disorder, social anxiety, panic disorders[146] and

occasionally PTSD[147]. SSRIs block the 5-HTT resulting in an increased level of 5-HT in the synaptic cleft[148-150]. The currently available SSRIs are citalopram (Cipramil®), escitalopram (Lexapro®), fluoxetine (Prozac®), fluvoxamine (Fevarin®), paroxetine (Seroxat®) and sertraline (Zoloft®). Some SSRIs, like fluvoxamine and fluoxetine have varying degrees of selectivity for the 5-HT, norepinephrine and dopamine transporters, whereas others (e.g. citalopram, escitalopram, paroxetine and sertraline) have high affinity for the 5-HTT and only weak affinity for the noradrenaline and dopamine transporters[151]. Besides the positive effects on affective disorders, clinical studies have shown that there are many side effects upon chronic administration of SSRIs, like sexual dysfunction[152], suppression of rapid eye movement sleep[153], nausea[154,155], decreased appetite[156] and aggression/violence[157,158], indicating that further optimization of chronic treatment of affective disorders is still needed. In addition, it takes weeks of treatment with SSRIs before clinical effects are noticed, which also needs optimization[159].

SSRI exposure during development

While numerous trials have shown robust safety of SSRIs and positive effects on treatment of affective disorders in adults, there are alarming studies showing that perinatal and adolescent SSRI exposure have negative effects on development and behavior. The highest risk for women to suffer from depression is during the childbearing years[160]. They may become pregnant while being treated for depression or may require antidepressants to fight depression during pregnancy[161-163]. Depression is associated with an increased risk of preterm delivery, low birth weight, higher impulsivity, maladaptive social interactions, and behavioral and emotional difficulties[164,165]. Treatment of depression is therefore important for these women and accordingly around 25% of the women treated for depression continue antidepressant use, and another 0.5% start with antidepressant drugs during the pregnancy, with SSRIs as the most frequently used antidepressants[161]. SSRIs can cross the placenta and are found in breast milk, thereby reaching the (unborn) child[166,167]. Studies have shown that perinatal SSRI exposure can increase the risk for neonatal withdrawal symptoms (including continuous crying, restlessness, fever, tremors, rigidity, respiratory distress, feeding difficulties, sleep disturbance, hypoglycemia, and seizures) which are temporary and are usually resolved within two weeks[168,169]. In addition, an increased risk for preterm birth[170], low birth weight[5,171], defects in motor behavior[172,173], autistic-like behavior as well as persistent pulmonary hypertension[174,175] and congenital heart defects[166,176] are observed in children perinatally exposed to SSRIs. Rodent studies investigating long-term effects of SSRIs showed that perinatal SSRI exposure increases the risk for anxiety and depression-like symptoms in adulthood[177-179]. Furthermore, there is increasing evidence that SSRI treatment during childhood and the adolescent period is associated with negative effects, such as increased risk for suicidal ideation, agitation, reduced social behavior[180], depression and anxiety[181-185]. However, it is important to keep in mind that in children and adolescents SSRIs also have beneficial effects[186-188]. Unlike fetuses, children and adolescents usually receive SSRIs for

the treatment of harmful mental states and in these situations no treatment at all will most likely lead to more severe health problems. Together, these data show that SSRIs exert age-dependent effects with negative outcomes during early-life.

5-HT function and signaling during neurodevelopment

The paradoxical negative effects of perinatal reduction in functional 5-HTT (perinatal SSRI exposure and S-allele carriers) compared to functional 5-HTT reduction only during adulthood (adult SSRI treatment), might be related to the neurotrophic function of 5-HT. 5-HT can promote neurodevelopmental processes like neuronal outgrowth, synaptogenesis and migration processes[189-193]. In addition, 5-HTT shows a more widespread distribution during neurodevelopment, which might contribute to the different effects observed after perinatal 5-HTT reduction compared to adult 5-HTT reduction[194]. During development 5-HTT is not only present on 5-HT neurons from the raphe nuclei, but also on non-serotonergic neurons. Mice studies show that at mid-gestation (embryonic day 10.5) expression of 5-HTT begins in the 5-HT neurons of the raphe nuclei, and subsequently emerges in the non-serotonergic neurons of the sensory system (thalamus, retina, somatosensory cortex) and corticolimbic regions (hippocampus, E14–E15). Expression in the PFC starts around birth. During the second postnatal week 5-HTT expression in non-serotonergic neurons ends rapidly[194]. It has been proposed that the transient broader 5-HTT expression during the perinatal period may serve to maintain stable 5-HT levels important for developmental processes. The thyroid hormone is shown to be involved in repression of the transient 5-HTT expression[195,196]. Studies in humans and non-human primates are limited, but show that in human embryos of gestational week 8–11 5-HTT is expressed in fibre tracts of the non-serotonergic internal capsule and optic tract[197], and at midgestation 5-HTT appears in all major sensory afferents (dorsal root ganglia, retinal ganglion cells, cochlear nucleus and olfactory nerves) in non-human primates[198]. Regions where the transient 5-HTT is co-expressed with 5-HT receptors could be important for brain development. 5-HTT is co-expressed with 5-HT_{2A} and 5-HT_{2C} in the mPFC and downstream pathways of these receptors are involved in dendritic maturation and cell death[199-201]. Furthermore, co-expression of 5-HTT and presynaptic 5-HT_{1B} is observed in the thalamocortical axons (TCAs)[202-205]. 5-HT_{1B} is linked to protein kinase A mediated signaling which can play a role in the response of axons to guidance factors, such as netrins and ephrins[206,207]. The transient broader expression of 5-HTT and the neurotrophic effects of 5-HT together ensure that the consequences of reduced functional 5-HTT can be different depending on the developmental stage of occurrence.

Neurodevelopment and altered 5-HT signaling

In order to unravel the developmental consequences of increased extracellular 5-HT, as seen in S-allele carriers and perinatal SSRI exposure, it is important to know what developmental processes occur during the neonatal stage and during infancy and adolescence. Environmental input and molecular events of gene expression changes are essential for normal brain

development, and disruption of either can fundamentally alter neural network formation, potentially leading to neurodevelopmental disorders (see Box 2). Here we give an overview of the main developmental processes taking place during fetal and postnatal brain development. In addition, the effects of 5-HT on several of these processes are mentioned. For comparison of experimental data between humans and rodents we have to take into account that rodents are born more immature compared to humans in terms of brain maturation[208]. See Table 2 for an overview of developmental processes across comparable ages in humans and rodents.

Neurodevelopment and altered 5-HT signaling during the human fetal period

Much of brain development in the human fetal period (gestational week 8 till birth) centers around the processes of neuron production, migration and differentiation. Beginning on embryonic day 42, cell division of neural progenitor cells located in the ventricular zone (VZ) begins to shift from symmetrical (two neural progenitor cells) to asymmetrical (one neural progenitor and one neuron)[209]. The new progenitor cell remains in the VZ and continues to divide, while the postmitotic neuron leaves the VZ to take its place in the developing neocortex. Because of the greater distances, neurons require radial glial guides to support their migration[210]. Radial glial guides (which are neural progenitor cells[211,212]) in the VZ extend a basal process that attaches to the pial surface of the brain, which forms a scaffold along which neurons can migrate. The earliest produced neurons migrate to the deepest cortical layers and subsequently migrating neurons migrate to successively more superficial layers creating an inside out order of migration[213]. Once the young neurons have reached their target region of the cortex, they start to develop axons and dendrites that allow them to communicate with other neurons. The growth cone, a dynamic, actin-supported extension of a developing neurite, is involved in axon branching and outgrowth[214] and samples the environment for guidance molecules. Guidance molecules direct the axon toward its target and can be attractive, signaling movement toward a source, or repulsive, guiding movement away from a source. Once the axon has reached its target, connections with the target cell are formed, called synapses, which are essential for communication in the nervous system.

During the fetal stage there is the period of transient widespread 5-HTT expression and 5-HT can influence fetal neurodevelopmental processes like neuronal outgrowth and migration[189-193]. As a consequence, deregulation of 5-HT signaling in this period might have a widespread effect on brain development. Rodent studies have shown that early-life SSRI treatment and genetic inactivation of 5-HTT result in defects in the somatosensory cortex and the corticolimbic system. In rodents the primary somatosensory cortex contains ''barrels'' in layer IV which correspond to functional inputs from whiskers on the contralateral snout in a one-to-one relationship[215]. The centre of a barrel contains projection targets of TCAs that conduct sensory information and form terminal clusters in layer IV. The 'walls' of the barrels consist of neurons in layer IV that organize around the terminal clusters from TCAs and receive synaptic contacts[194]. Early-life SSRI treatment and genetic inactivation of 5-HTT causes impaired innervation of cortical layer IV by reduced arborisation (formation of a

treelike shape) of TCA clusters, diffuse barrel patterns and dendritically smaller spiny stellate cells with a reduced spine density in the somatosensory systems[194,205,216,217]. A corticolimbic circuit regulated by the serotonergic system is the connection between the dorsal raphe nucleus, mPFC and the amygdala, which is involved in stress-related reactions[80,218-220]. Genetic inactivation of 5-HTT leads to increased dendritic spine densities in pyramidal cells of the amygdala and abnormally increased dendritic branching in pyramidal neurons of the PFC[194,221].

Box 2: Neurodevelopmental disorders

Neurodevelopmental disorders are psychiatric conditions originating in childhood that involve serious impairments in different areas of the central nervous system. The main neurodevelopmental disorders are autism spectrum disorder (ASD), intellectual disability (ID), schizophrenia (SCZ) and attention deficit (hyperactivity) disorder (AD(H)D). ASD is a disorder characterized by impairments in three core domains; reciprocal social interaction, communication (verbal and nonverbal) and restricted and repetitive behavior. ID is characterized by significant limitations in both intellectual functioning (e.g. learning, reasoning, problem solving) and in adaptive behavior (conceptual, social, and practical skills that are learned and performed by people in their everyday lives). SCZ is characterized by delusions and hallucinations and by poor emotional responsiveness, and disorganized thinking and speech. AD(H)D is characterized by inattention, hyperactivity/impulsivity and motivational/emotional dysregulation. All four neurodevelopmental disorders are associated with disturbed developmental processes, such as proliferation of neuronal progenitor cells (ASD, ID, SCZ), migration of neurons (ID, ASD), axon guidance (ASD, AD(H)D), synaptogenesis (ASD, ID, AD(H)D) and pruning (SCZ)[222].

Neurodevelopment and altered 5-HT signaling during postnatal neurodevelopment

In the human postnatal period, neurogenesis continues to take place in a very limited degree and is restricted to two brain regions, the subventricular zone (SVZ, lies adjacent to the VZ) and the subgranular zone (SGZ) in the DG of the hippocampus. At gestational week 7–8 the SVZ forms above the VZ[223,224]. In this zone new neurons continue to emerge and migrate in the direction of the olfactory bulb, where they differentiate into interneurons. In the DG the newly formed neurons migrate from the SGZ only as far as the nearby granular layer[225]. These two forms of neurogenesis appear to continue throughout adult life but produce only a small percentage of the neuronal population[226]. The functional relevance of adult neurogenesis is uncertain[227], but there is evidence that hippocampal adult neurogenesis is important for learning and memory[228]. Many factors may affect the rate of hippocampal neurogenesis. Exercise and an enriched environment have been shown to promote the survival of neurons and the integration of newborn cells into the existing hippocampus[229,230]. On the other hand, chronic stress and aging can result in a decreased neuronal proliferation[226,231]. It has been shown that 5-HT depletion reduces the rate of neurogenesis in the adult DG, whereas increased levels of 5-HT and activation of 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} receptors increase neurogenesis in the DG[232]. Adult neurogenesis in the DG has been shown to be reduced

in depressed patients and can be increased by 5-HT-related antidepressants (SSRIs, MAO inhibitors)[30].

In contrast to neurogenesis, gliogenesis (the proliferation and migration of glial progenitors) occurs largely in the postnatal period. Glial progenitors proliferate in the forebrain SVZ and migrate outwards into other brain regions. Subsequent differentiation of glial progenitors results in function-specialized glial lineages consisting of astrocytes, oligodendrocytes, ependymal cells and microglial cells[233]. The peak in overall gliogenesis occurs around birth (with each specific glial lineage having its own peak at a different time point) and gliogenesis continues until adulthood. Astrocytes, the most abundant type of glial cells in the CNS, regulate the external chemical environment of neurons by removing excess potassium ions, and by recycling neurotransmitters released during synaptic transmission[233]. Ependymal cells line the spinal cord and the ventricular system of the brain. These cells are involved in the creation, secretion and circulation of CSF and make up the blood-CSF barrier[234]. Oligodendrocytes produce myelin, which insulates axons to facilitate electric signal transduction (Box 3)[233]. Finally, microglial cells are specialized macrophages capable of removing cellular and foreign debris within the CNS[233]. Glial progenitors persist indefinitely in the adult brain in a wide anatomical distribution, and can differentiate in response to injury[235]. There are a few studies showing an effect of 5-HT on glial cell development. Oligodendrocyte development and myelination can be influenced by 5-HT levels *in vitro*[236]. Furthermore, myelin sheath formation is disturbed in rats perinatally exposed to SSRIs, with the strongest effects seen after postnatal exposure (Postnatal day (PND)8–21)[237]. Postnatal SSRI treatment has been shown to affect astrocyte development, with the strongest effects seen after treatment during the peri-adolescence period (PND 21-35)[238,239].

Another process that continues after birth is synapse formation. The cerebral cortex produces most of its synaptic connections after birth in a massive burst of synapse formation known as the exuberant period, which is important for the formation of brain networks[240]. This period of synaptic exuberance varies in different parts of the cerebral cortex. Synaptic density peaks in the human primary visual cortex as early as 8–12 months of age, compared to 2–4 years of age in the PFC[241]. The number of synapses plateaus at levels nearly twice as high as those observed in the adult brain, and then gradually declines to adult levels across the period of childhood and adolescence[242]. Every experience excites certain neural circuits and leaves others inactive. Those that are consistently turned on over time will be strengthened, while those that are rarely excited will be eliminated[243]. The elimination of unused synapses is called synaptic pruning. Synaptic pruning streamlines children's neural processing, making the remaining circuits work more quickly and efficiently. The pruning process is completed first in primary sensory regions and last in frontal and parietal regions[244]. 5-HT is involved in the regulation of synaptogenesis and synaptic pruning[191,245,246]. 5-HT can disturb pruning by its capability to inhibit the activation of microglia via 5-HT_2 receptor activation, resulting in a reduced uptake of synaptic particles[247]. 5-$HTT^{-/-}$ mice display increased spine densities during adulthood, which can be a result from pruning deficits[248].

Table 2. Developmental processes across comparable ages in humans and rodents

Human	Rodent	Developmental milestone
23-32 wk gestation	PND1-3	Establishment of the blood-brain barrier[249,250] Immune system development[251]
36-40 wk gestation	PND7-10	Peak brain growth spurt[252,253] Peak in gliogenesis[254,255] Increasing axonal and dendritic density[253,256,257] Oligodendrocyte maturation and onset of myelination[258] Consolidation of immune system[251]
0-1 year old	PND12-13	End of transient wide-spread 5-HTT expression[194]*
2-3 year old	PND20-21	Brain reaches 90-95% of adult weight[252,259,260] Peak in synaptic density at 50% > adult levels[261,262] Peak in myelination rate[263]
4-11 year old	PND25-35	Fractioning/specialization of prenatal cortex neural networks (structural maturation)[264] Maximum volume of grey matter and cortical thickness[265,266]
12-18 year old	PND35-49	Reduced synapse density, reaching a plateau at adult levels[261,267] Ongoing myelination: increasing white matter volume[257,259] Refinement of cognitive dependent circuitry (by pruning and myelination)[268]
20 + year	PND60+	Adult levels of synaptic density[261] Ongoing myelination and declining grey matter[269,270]

Note: Table adjusted from Semple and colleagues[241]. PND: postnatal day. * Based on rodent studies.

The 5-HTT knockout rodent

Studying behavior and gene expression across development is time-consuming and rather complicated in humans since their life-time is relatively long and environmental factors cannot be controlled very well. To circumvent these limitation, rodents are often used for these kind of studies. The 5-HTT knockout (5-HTT$^{-/-}$) rat and mouse are frequently used for studying the effects of increased 5-HT signaling. Both 5-HTT$^{-/-}$ rats and 5-HTT$^{-/-}$ mice show reduced 5-HT uptake (synaptosomes and primary neurons)[271-273] and increased extracellular 5-HT levels across the brain[271,274-276]. In addition, reduced firing of raphe neurons, decreased expression and sensitivity of 5-HT$_{1A}$ inhibitory autoreceptors and increased 5-HT$_{1B}$ receptor sensitivity have been observed in 5-HTT$^{-/-}$ mice and rats[274,277-280]. 5-HTT$^{-/-}$ rats and mice show a rather mild phenotype, they reach normal age (Homberg, unpublished findings) and show normal coat and whisker condition[281]. Phenotype differences are present in both 5-HTT$^{-/-}$ mice and rats, but are not often readily apparent at first glance. Both 5-HTT$^{-/-}$ rats and 5-HTT$^{-/-}$ mice show anxiety and depression-like behavior. They show anhedonia and behavioral despair[179,282], as reflected by reduced sucrose preference and increased immobility in the forced swim test[126,233281]. Increased anxiety-like behavior is observed in the elevated plus-maze, open field, light dark exploration and novelty suppressed feeding test[177,179,282,283]. The anxiety and depression-like behavior observed in these 5-HTT$^{-/-}$ rodents resembles the effects seen in the 5-HTTLPR S-allele carriers and in early-life SSRI exposed humans and rodents.

Both 5-HTT$^{-/-}$ rats and 5-HTT$^{-/-}$ mice display reduced social behavior[284,285]. With regard to cognitive functioning, 5-HTT rats show impaired object recognition, but show better strategy set-shifting and perform better in a rodent version of the Iowa gambling task[286-288]. In addition, both 5-HTT$^{-/-}$ rats and 5-HTT$^{-/-}$ mice show improved reversal learning[289-291]. There are no studies that have investigated behavior across developmental stages in 5-HTT$^{-/-}$ rodents.

Box 3: Myelination

Oligodendrocyte progenitor cells (OPC) are the glial progenitors important for myelination, which is the production of a fatty white substance that surrounds the axon, called myelin. Upon reaching their destination, OPCs begin to differentiate by extending processes and increasing myelin protein expression. The processes then begin to form membrane wraps around nearby axons. Between gestational weeks 20 and 28 in humans, mature myelin is detected first in subcortical regions and later in cortical regions. Myelin enhances the speed and fidelity of the transmission of information encoded in action potentials that propagate along neurons[292]. Because myelination of an axon has dramatic functional consequences for how fast neurons will transmit their signals, myelination is likely to have a role in modulating network activity in the brain[293]. There is evidence that myelin sheath formation is regulated by experience during development and also in adult life[294-296]. The dynamic extent of myelin may serve as a form of plasticity to adapt brain function to environmental stimuli. Structural components of CNS myelin are myelin basic protein (MBP), proteolipid protein (PLP), myelin associated glycoprotein (MAG), myelin and lymphocyte protein (MAL), myelin oligodendrocyte glycoprotein (MOG) and 20,30-cyclic nucleotide 30-phosphodiesterase (CNPase)[297,298], with MBP and PLP as the most abundant proteins[167,168]. These components are important for myelin formation and myelin compaction[298]. In addition, several other components are associated with oligodendrocyte differentiation and myelination, like amongst others, oligodendrocyte transcription factor 1 and 2 (OLIG1/OLIG2), Sry-related HMG-Box gene 10 (SOX10)[299,300], ciliary neurotrophic factor (CNTF)[301] and transferrin (TF)[302].

Genome-wide gene expression analysis

While several studies reported behavioral and structural consequences of 5-HTT down-regulation during development, the molecular mechanisms contributing to these developmental changes are poorly understood. Studying gene expression during development might reveal important new insights and might potentially lead to new targets for treatment of 5-HT-related disorders. Gene expression analysis can be performed for specific genes using quantitative PCR techniques or genome-wide using RNA sequencing (RNA-seq). Since several chapters in this thesis include RNA-seq experiments, the RNA-seq procedure and transcriptional changes that can be detected with this technique will be explained here. For RNA-seq, messenger RNA (mRNA) is isolated from brain tissue and converted into complementary DNA (cDNA). Subsequently, this cDNA is fragmented and adapters are ligated to the fragments. Fragments around 300 base pairs are selected and used for sequencing. Figure 3 shows the (Illumina) sequencing steps that occur in the sequencer. The sequences of these fragments (reads) are mapped to the reference genome and the number of fragments

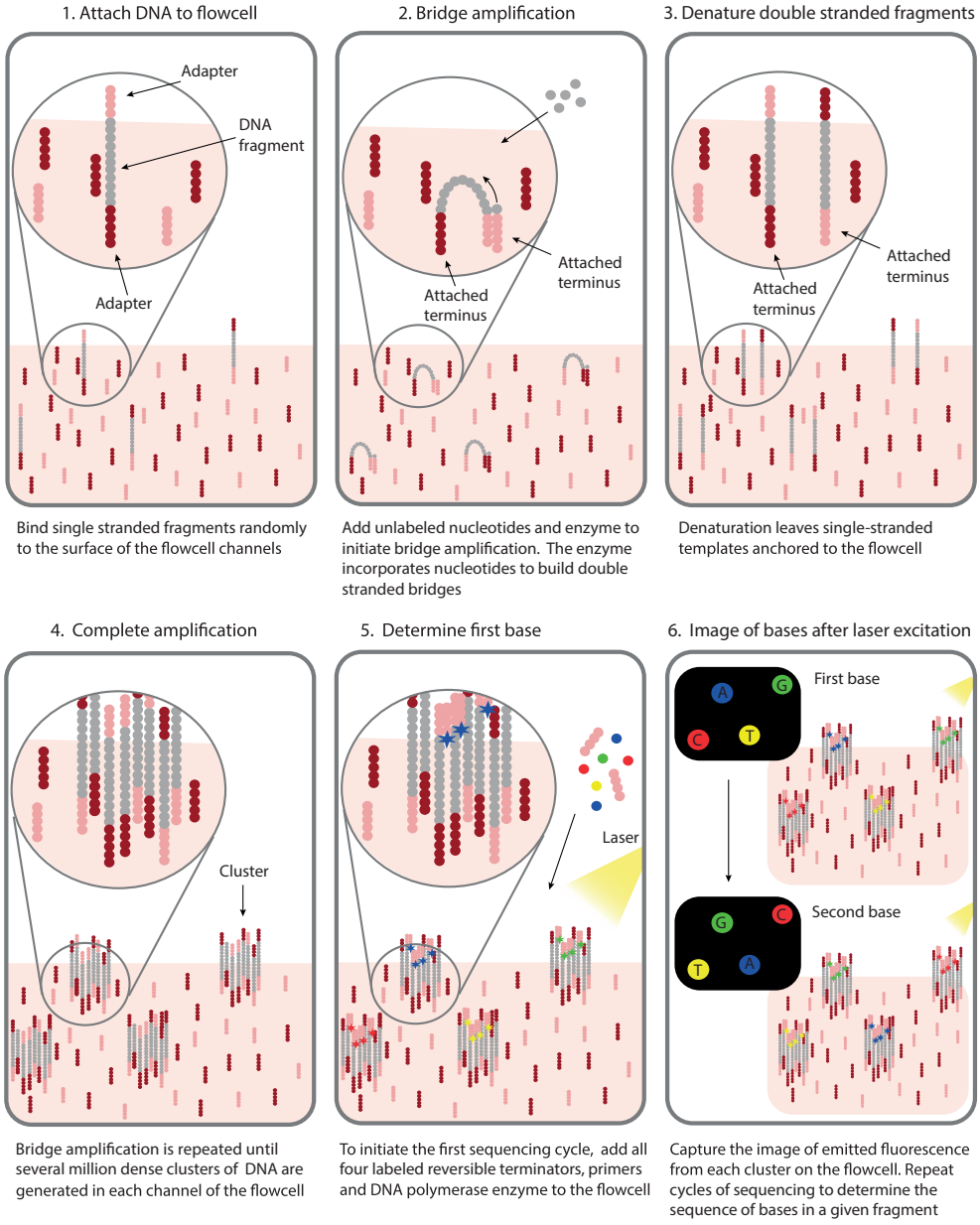

Figure 3. Illumina (Solexa) sequencing. Basic processes of sequencing (adapted from https://www.illumina.com/documents/products/techspotlights/techspotlight_sequencing.pdf).

per kilobase per million mapped reads is calculated per gene or exon. The more reads mapped to a specific region, the higher the expression of that region. Differences in gene expression between developmental time points or different genetic models can be analyzed. Expression of protein-coding genes as well as expression of ncRNAs can be analyzed using RNA-seq. Protein-coding genes, which only account for a minority of cellular transcriptional output[303], are the best annotated genes and the function of the majority of these genes is known. Non-coding RNAs are less well annotated and their function is often unknown. In the RNA-seq method described above there is a selection for 300 bp fragments and therefore small ncRNAs, such as most of the miRNAs, are not detected using this method. Another transcriptional mechanism that can be investigated by RNA-seq is alternative exon usage, which is a commonly used mechanism for increasing coding diversity within genes. In this process, particular exons of a gene may be included within or excluded from the final, processed mRNA. Consequently, the proteins translated from alternatively spliced mRNAs will contain differences in their amino acid sequence and, often, in their biological functions. Alternative exon usage comprises alternative promoter usage (5' exon), alternative splicing of internal exons and alternative 3' exon usage.

Aim and outline of the thesis

The aims of this thesis are to elucidate the transcriptional changes occurring across postnatal mPFC development and to unravel the behavioral and molecular consequences of aberrant 5-HT signaling across development in the absence of adverse environmental conditions. Behavior and morphological consequences of aberrant 5-HT signaling are the main focus of most studies and the amount of molecular studies is still limited. While most molecular studies focus on the effects of SSRIs on gene expression immediately after the last administration (short-term/ acute effects), the long-term effects of adult SSRI exposure on (genome-wide) gene expression are unclear. In addition, the paradoxical effects of SSRI exposure during early-life and adulthood on adult anxiety and depression-like behavior would suggest that adult gene expression is affected in opposite direction after early-life compared to adult SSRI exposure, a presumption that remains to be investigated. Furthermore, there is ample evidence that perinatal exposure to SSRIs (causing pharmacological blockade of 5-HTT) has negative effects on behavior in adulthood, but besides the transient withdrawal symptoms, information about the effect on the development of CNS-controlled behavior in early-life and their long-term consequences is lacking. Data about development of CNS-controlled behavior is also missing for individuals with inherited 5-HTT down-regulation. In addition, gene expression analysis at different developmental time points in a model of reduced functional 5-HTT expression will contribute to linking gene expression to the observed behavioral changes.

In **chapter 2**, we summarized the effects of SSRIs on gene expression in the brain. The main groups of genes affected by SSRIs are genes encoding serotonin receptors, components of non-serotonergic neurotransmitter systems, neurotrophic factors, hypothalamic hormones and inflammatory factors.

In **chapter 3**, we investigated whether the paradoxical effect of SSRI exposure during early-life and adulthood on adult anxiety and depression-like behavior is also observed on gene expression level. We treated rats with fluoxetine or methylcellulose (control group) at adulthood and measured anxiety-like behavior in the novelty suppressed feeding test. We have performed genome-wide gene expression analysis in the hippocampus of these rats 40 days after fluoxetine treatment and found several differentially regulated myelin genes. In addition, we examined the expression of several myelin genes in the hippocampus of adult rats postnatally (PND1-21) exposed to fluoxetine using reverse transcription quantitative PCR (RT-qPCR).

In **chapter 4**, we investigated the effects of reduced 5-HTT expression during early-life on behavior across development. We performed several behavior experiments in rats perinatally exposed to fluoxetine and rats perinatally exposed to methylcellulose (control group). In addition, we performed the same tests in 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats. Behavior experiments included body weight, eye opening, motor-related behavior, reflex development, olfactory function, grooming behavior, sensorimotor gating, object directed behavior and novel object recognition, and were performed during the first three postnatal weeks and repeated during adolescence and adulthood if possible.

In **chapter 5**, we examined expression changes during postnatal development in the PFC, a region frequently involved in affective disorders. We performed genome-wide expression analysis in the mPFC at five developmental time points from infancy till adulthood and analyzed developmental changes in coding-gene expression, long intergenic non-coding RNA expression and alternative exon usage. We provide a data resource of temporal gene expression in the developing mPFC from infancy to adulthood.

In **chapter 6**, we investigated the molecular mechanisms contributing to the structural and behavioral changes in the mPFC related to inherited 5-HTT down-regulation. We examined differentially expressed coding-genes in the mPFC of 5-HTT$^{-/-}$ rats compared to 5-HTT$^{+/+}$ rats at five time points across postnatal development. In addition, DNA (hydroxy) methylation levels (5(h)mC) were measured at the same five developmental time points.

Finally, **chapter 7** provides a general discussion and future perspectives regarding the research described in this thesis.

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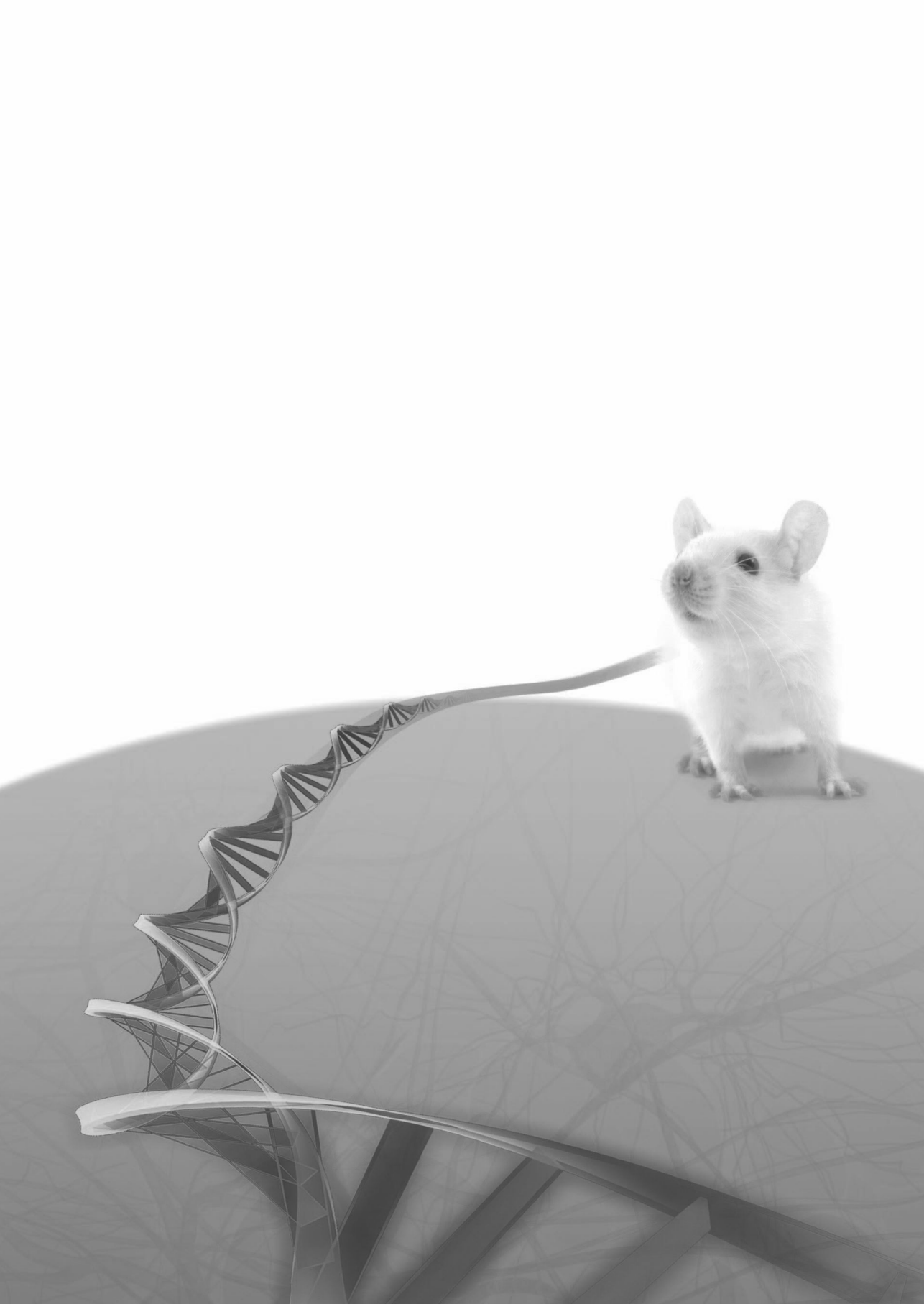

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The genetics of selective serotonin reuptake inhibitors

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Abstract

Selective serotonin reuptake inhibitors (SSRIs) are among the most widely prescribed drugs in psychiatry. Based on the fact that SSRIs increase extracellular monoamine levels in the brain, the monoamine hypothesis of depression was introduced, postulating that depression is associated with too low serotonin, dopamine and noradrenaline levels. However, several lines of evidence indicate that this hypothesis is too simplistic and that depression and the efficacy of SSRIs are dependent on neuroplastic changes mediated by changes in gene expression. Because a coherent view on global gene expression is lacking, we aim to provide an overview of the effects of SSRI treatment on the final targets of 5-HT receptor signal transduction pathways, namely the transcriptional regulation of genes. We address gene polymorphisms in humans that affect SSRI efficacy, as well as *in vitro* studies employing human-derived cells. We also discuss the molecular targets affected by SSRIs in animal models, both *in vivo* and *in vitro.* We conclude that serotonin transporter gene variation in humans affects the efficacy and side effects of SSRIs, whereas SSRIs generally do not affect serotonin transporter gene expression in animals. Instead, SSRIs alter mRNA levels of genes encoding serotonin receptors, components of non-serotonergic neurotransmitter systems, neurotrophic factors, hypothalamic hormones and inflammatory factors. So far little is known about the epigenetic and age-dependent molecular effects of SSRIs, which might give more insights in the working mechanism(s) of SSRIs.

Introduction

Selective serotonin reuptake inhibitors (SSRIs) are among the most widely prescribed drugs in psychiatry. Besides serving as antidepressants, they are also used in the treatment of anxiety-related disorders (obsessive compulsive disorder, social anxiety, panic disorders)[1], autism[2], eating disorders[3], and occasionally posttraumatic stress disorder[4]. SSRIs increase the extracellular serotonin (5-hydroxytryptamine; 5-HT) levels in the synaptic cleft by blocking the serotonin transporter (5-HTT), a Na^+/Cl^- inward rectifying transporter[5] located on presynaptic nerve terminals. This increase in extracellular levels of 5-HT is consistently shown in several brain regions by performing microdialysis followed by high-performance liquid chromatography[6-11]. The amount of increase can differ between studies, which might be caused by differences in SSRI treatment, brain region or timepoint of measurement. For example, it is shown that acute SSRI treatment results in a transient 5-HT increase, while repeated treatment increases baseline 5-HT levels[8]. There is no evidence that the SSRI-induced increase in extracellular serotonin is caused by blocking the serotonin break down or increasing serotonin synthesis. Due to negative feedback mechanisms caused by activation of inhibitory autoreceptors (discussed below), serotonin synthesis and release is even decreased[7]. Some SSRIs (fluvoxamine (Luvox), fluoxetine (Prozac)) have varying degrees of selectivity for the 5-HTT, norepinephrine and dopamine transporters, whereas others (e.g. citalopram (Celexa), s-citalopram (Lexapro), paroxetine (Paxil), and sertraline (Zoloft)) have high affinity for the 5-HTT and only weak affinity for the noradrenaline and dopamine transporters[12]. Based on the fact that SSRIs increase extracellular 5-HT levels in the brain, the monoamine hypothesis of depression was introduced, postulating that depression is associated with too low 5-HT (and dopamine and noradrenaline) levels[13]. This hypothesis has governed the depression field for several decades. However, there is accumulating evidence that this hypothesis is too simplistic**.** For instance, SSRI treatment results in desensitization of the 5-HT_{1A} autoreceptors and thereby cause an increase in raphe firing[14]. In both animal and clinical studies it has been found that SSRI-5-HT_{1A} antagonist co-administration leads to enhanced antidepressant responses[15,16]. A recent study has also shown that a 5-HT_{1A} receptor siRNA conjugated to the SSRI citalopram had antidepressant effects in mice[17]. These findings suggest that the antidepressant effects of SSRIs at least depend on 5-HT_{1A} receptor function. The process is rather complex and probably goes beyond the serotonin system. For instance, 5-HT_{1A} receptor activation results in a decreased release of glutamate[18]. The monoamine hypothesis is also not in line with the finding that depletion of the 5-HT precursor tryptophan does not induce depression-like mood symptoms in healthy subjects[19].

In addition, there are several lines of evidence from rodent studies that *in utero* or neonatal exposure to SSRIs leads to paradoxical autism-, anxiety- and depression-like symptoms in later life[20]. There is evidence that the 5-HTT expression pattern at early developmental stages is more widespread and it is shown that early life SSRI treatment can affect neurodevelopment[21]. Both, more widespread 5-HTT expression and neurodevelopment, can contribute to the paradoxical effects, however the exact mechanisms causing the differences

at behavioral level are still not clear. Furthermore, SSRIs increase risks of "possible suicidal ideation and suicidal behavior" by about 80%, and of "agitation and hostility" by about 130% in children with major depressive disorder (MDD)[22]. Consistent with these observations, animal experimental studies have shown that adolescent SSRI exposure leads to anxiety- and depression-related symptoms[23,24]. Taken together, a massive amount of data aiming to elucidate the working mechanism(s) of SSRIs has been generated, but simultaneously the findings have raised more questions than they answered. While it is clear that an increase in 5-HT per se does not increase mood state, it is still obscure what mechanisms do contribute to the therapeutic and side effects of SSRIs. Understanding these mechanisms is not only of high interest from a fundamental point of view, but also will lead to improvement of the treatment of disorders characterized by mood disturbances.

To understand SSRI mechanisms it is critical to comprehend 5-HT-mediated signaling. Up to now, 16 different 5-HT receptor subtypes have been identified: 5-$HT_{1A, 1B, 1C, 1D, 1E, 1F}$, 5-$HT_{2A, 2B, 2C}$, 5-$HT_{3A, 3B}$, 5-$HT_{4}$, 5-$HT_{5A, 5B}$, 5-$HT_{6}$ and 5-HT_{7}. All of them are G-protein coupled receptors, except for the 5-HT_{3A} and $_{3B}$ receptors which are ligand-gated ion channel receptors[25]. The presynaptic 5-HT_{1B} receptor is expressed in rodents and auto-regulates 5-HT release, while the 5-HT_{1D} receptor fulfils this purpose in humans[25]. The presynaptic inhibitory 5-HT_{1A} autoreceptor is located in the raphe nuclei and regulates the firing of serotonergic raphe neurons that project to widespread regions in the brain, as well as to the spinal cord. The 5-HT_{1A} and 5-$HT_{1B/D}$ receptors, as all other 5-HT receptors, are also found postsynaptically. Since SSRIs have indirect (increasing serotonin) and direct (SSRI dependent affinity for receptors like 5-HT_{2}[26] and 5-HT_{1A}[27]) effects on 5-HT receptors, 5-HT receptor agonists/antagonist are widely used to determine via which 5-HT receptor an SSRI effect is caused. Each receptor drives specific intracellular signalling pathways, targeting transcription factors to regulate transcription of multiple genes involved in components of the serotonergic and other neurotransmitter systems as well as in neurotrophic and developmental functions. In addition, 5-HT-receptor linked intracellular signalling pathways may target epigenetic processes like DNA methylation and histone acetylation/methylation. These epigenetic modifications influence the transcriptional machinery, and thereby provide another means by which SSRIs can affect a wide array of central processes. Given the multiple 5-HT receptors and their coupling to various signal transduction pathways and genomic targets (e.g. transcription regulators), which change across developmental stages and in response to external stimuli like stress or pharmacological agents, it may not be surprising that the mechanisms underlying SSRI effects are far more complex than the monoamine hypothesis suggests.

Here we aim to provide an overview of the effects of SSRI treatment on the final targets of 5-HT receptor signal transduction pathways, namely the transcriptional regulation of genes (Table 1). We address gene polymorphisms in humans that affect SSRI efficacy, as well as *in vitro* studies employing human-derived cells. We also discuss the molecular targets affected by SSRIs in animal models, both *in vivo* and *in vitro* (see Box 1 for the (dis)advantages

Table 1. SSRIs with their target genes

SSRI	Serotonergic genes	Other neuro-transmitter genes	Neuroplasticity genes	Hypothalamic genes	Inflammatory genes	Miscellaneous
Fluoxetine	SLC6A4[28-33],GCHFR[34] , TPH2[35], HTR1A[36-38], HTR2A[39,40], PDE11A[41,42], Slc6a4[43,44], Tph2[43-45], Htr1a[14], Htr1b[14,46], Htr2c[47,48], Htr7[49], Prkcd[50], Prkcg[50], Mapk9[50], Prkcb[50], Jak1[50], Pfkm[50], Pde4a[51-53], Pde4b[52,53], Pde4d[53], Pde3b[54], Adcy1[54], Adcy2[54], Creb1[55-57], Mapk1[57], Ccbl1[58], Aadat[58], Kmo[58]	ADRBK1[59], ADRB1[60], GSK3B[61], COMT[26], GNB3[37,38], Adrb1 [62], Drd3[63], Th[45,64-67], Dbh[67], Dbh[68], Gabrg2[69], Gabbr1[70], Gabbr2[70], Grik2[71,72], Grik1[72], Gria2[72] , Gria4[72], Chrna5[73], Chrnb4[73], Slc17a7[74,75], Gal[76]	BDNF[77,78], NGFR[79], SERPINE1[80], Bdnf[81-84], Arc[81,85], Nrn1[85], Nptx2[85], Klf10[85], Ankrd1[85], Arl4d[85], Syp[65,86], Gap43[86], Kv4.2[87]*, Npas4[88], Serpini1[89], Reln[90], Igf1[91], L1cam[92]	CRHR1[93,94], Avpr1b[95], Avp[95], Oxt[95], Crh[45,64], Nr3c1[45,96-98], Nr3c2[64,96,98,99], Pomc[100], Mc4r[100], Npy[101,102], Mchr1[103], crf1[104], npy[104], cartpt[104], esr2[105], esr1[105], it[105]*	IFNG[106], IL1B[84]	Gata6[107], Ttr[107], Afp[107], Alb[107], Gfap[65], olig2[65], Plp1[65], S100b[65,108], Tuj1[65], Nurr1[65], miR-16[108], Ccnd1[109], Adar2[71], Bcl2l1[110,111], Bcl2[112], Bax[112], Brs3[73], Qrfpr[73], Mtor[57], Mecp2[113], Mbd1[113], Hdac2[113], Per1[114], Per2[114], Clock[114], Arntl[114], Cry1[114], Npas2[114], Fos[115], zif268[115], Rabif[116], Bzrap1[117], Aanat[118,119], Foxg1[120], Hes1[121], Hes5[121], Jag1[121], Notch1[121]
Citalopram	SLC6A4[30,32,122-124], TPH2[35,125], TPH1[126], HTR1A[127], HTR1B[127], HTR2A[39,128-130], HTR2C[131], IDO2[132], MTHFR[133], Tph2[134], Htr1a [135], Htr7[49], Prkcd[31], Prkcg[31], Mapk9[31], Prkcb[31], Jak1[31], Pfkm[31], Adcy1[136], Crebbp[56,134], Ccbl1[58], Aadat[58], Kmo[58]	ADRBK1[137], COMT[116,117], GSK3B[61], GRIA3[138] , GRIK2[138], GRIA1[138], GRIN3A[138], GLDC[139], SLC6A3[120], Drd1[140], Drd2[141], Grin1[142], Grin2a[142], Grin2b[142]	BDNF[133,143] , NGFR[79], PAI-1[80], DTNBP1[144], Bdnf[145], Dlg4[146]	CRHR2[147], CRHBP[148], FKBP5[149], Pomc[150-152], Crh[152]		ABCC1[153], OPRM1[154], CACNA1C[155], Sv2b[134], Ndrg2[134], Eno2[134], Sst[156], Wfs1[157]
Sertraline	SLC6A4[33,158], HTR1A[159], HTR2A[40], Tph[160], Creb1[55]	GNB3[38], Dbh[68], Drd1[161], Gad1[162]	BDNF[163], Bdnf[82,164], Ntrk2[164] , Serpini1[89]		TNFSF8[165], IL2RA[165], LMAN1[165], STAT1[165]	TFDP1[165], SLC16A1[165], MAD2L1[165], MSH2[165], BUB1[165], ACTB[165], CTSD[165], ITGA5[165] , CDC2[165], CDC6[165], CDKN1B[166], CDKN1C[166], S100A10[166], ARRB2[166], GADD45B[166], Ndrg2[167], Rnf103[168], Fos[169]
Paroxetine	SLC6A4[28,30,32,170-173], HTR1A[159], HTR2A[39,40,174,175], HTR3B[176,177], TPH1[178], TPH2[125], MAOB[179], CREB[180], Htr1b [46], Htr7[49]	ADRB1[60], GABRA4[181], GNB3[38,163], Dbh[68], Slc17a7[74]	BDNF[182], CHL[181], CCL5[181], ENDOD1[181], FLT1[181] , GAP43[181], SPRY2[181], Bdnf[183-185], Vegfa[184], Vgf[184], Arc[186]	Nr3c1[187]	TNFSF8[165], IL2RA[165], LMAN1[165], STAT1[165], IL1B[170]	CLOCK[188] , MCTP2[181], ENPP2[181], ARRB1[181], DDX60[181], DDX60L[181], ABCB1[189,190], HDAC5[180], CDC2[165], CDC6[165]
Escitalopram	SLC6A4[191-194], Creb1[195]	ADRBK1[59], GLDC[139], COMT[117]	BDNF[196,197], BDNF[198], Bdnf[195,199], Ntrk2[199], Carf[195]		IL11[200]	ABCB1[201], S100a10[202], Dnmt1[202], Dnmt3a[202]
Fluvoxamine	SLC6A4[28,203-205], HTR1A[206], HTR2A[40,174,175,207], MAOA[208], GNB3[209], Htr2c[210], Gnai2[211]	COMT[212], Th[66]	BDNF[78], Bdnf[213], Serpini1[89]			CLOCK[188,214], CYP2D6[207], Nos2[215]

Note: Genes harboring SNPs interacting with SSRIs and genes showing changes in mRNA expression after SSRI treatment are included (changes in protein levels are not shown). All genes are depicted in official gene symbol. * mRNA transcript, no official gene symbol available. Black, human; Blue, rodent; Green, fish. Underlined, based on polymorphisms; Double underlined, based on knockout studies; no line, based on mRNA expression.

of the biological materials). By presenting data into pathways, an overview is generated of pathways affected by SSRIs that could help to elucidate the working mechanisms of SSRIs. In addition, we propose a few research directions which might give more insights in the working mechanisms of SSRIs.

Box 1: Biological material
Studies addressing the working mechanisms of SSRIs are performed in humans and animals (e.g. rodents, fish) both *in vivo* and *in vitro*. Each of these approaches has its own advantages and disadvantages. Given that gene expression is highly tissue specific, experimental animals provide the advantage of the accessibility of the brain. Moreover, SSRI treatment (dosage, duration, age of treatment, etc.) and environmental conditions can be more tightly controlled. On the other hand, behavioral models for depression are debated because of their lack of construct validation, thus a clear link to neurobiological substrates. Although experimental animals provide the advantage of the accessibility of the brain, dissected brain areas are still very heterogenous regarding cell type. Cell systems overcome this caveat, although *in vitro* findings require validation *in vivo*. Furthermore, there are obvious ethical limitations with using human brain tissue. An exception would be the use of postmortem brain tissue, but this tissue delivers 'static' information, whereas gene expression and regulatory mechanisms are dynamic. To overcome these limitations peripheral cell lines have been used. In addition, there are a lot of studies on the association of gene polymorphisms in humans to SSRI efficacy.

Serotonergic system

Since the serotonergic system (Box 2 and Figure 1) is the main target of SSRIs, polymorphisms in genes involved in this system likely influence SSRI responsiveness. Furthermore, studies in animals (*in vivo* as well as *in vitro*) also show interactions between SSRIs and the serotonergic system. Below, we will describe the effects of SSRIs on the 5-HTT gene, 5-HT receptor genes and genes involved in 5-HT synthesis and downstream signaling pathways.

Human-polymorphisms

The serotonin transporter

One genetic factor modifying SSRI effects is the common serotonin transporter promoter polymorphism (5-hydroxytryptamine transporter-linked polymorphic region; 5-HTTLPR). The short (S) allele is associated with reduced transcription of the *SLC6A4* gene compared to the long (L) allele. The L-allele bears an A/G single nucleotide polymorphism (SNP), resulting in the La (L-like) and Lg (S-like) allelic variants[216]. In the treatment of generalized anxiety disorder it was found that the SSRI escitalopram had no efficacy in the La^- group *versus* moderate efficacy in the La^+ group, due to a higher placebo response in La^- subjects[191]. Thus, the 5-HTTLPR S-like allelic variants appear to be associated with poor SSRI responsiveness. This was confirmed by several other studies using SSRIs in Korean[33,217], Chinese[29,30], Japanese[205] and Caucasian[28,31,32,122,170,171,192,203,204] depressed patients, particularly in females[172,173,218]. Moreover, S-allele carriers were found to respond worse to fluoxetine in personality disorder

patients characterized by impulsive-aggressive behavior[219], and SSRI treatment in healthy subjects diminished the perception of sad and fearful pictures, but only in 5-HTTLPR L-allele carriers[193]. These and other studies have been evaluated in several meta-analyses and reviews of Kato and Serretti[220], Serretti and coworkers[221-223], and Weizman and Weizman[224]. Furthermore, a meta-analysis by Porcelli and colleagues[225] suggested an ethnic-specific effect of mixed antidepressants in which the Asians show a smaller effect of 5-HTTLPR on remission and response rates compared to Caucasians. The overall poor responsiveness to SSRIs in S-allele carriers may relate to reduced 5-HTT availability, but no clear association has been reported between 5-HTT genotype and central 5-HTT binding[226]. Alternatively, it may be explained by increased sensitivity to SSRI-induced side effects[227,228]. For instance, evidence shows that Dutch depressed patients with the 5-HTTLPR S/S or S/L genotype exhibit an increased risk of adverse events in response to SSRI treatment, especially general adverse events (dermatologic reactions, weight change and fatigue)[229].

Yet, several studies could not find an association between the 5-HTTLPR, either two- or tri-allelic, and SSRI responsiveness[159,173,230,231]. Potentially, differences in types of SSRIs, length of drug treatment, dose, evaluation methods, etc. may explain such discrepancies. Finally, no association was found between a variable number of tandem repeats (VNTR) in the second intron of the *SLC6A4* gene (STin2) and SSRI efficacy in depressed patients[173,232], although Chinese[30] and Asian patients[28] with the STin2 12/12 genotype – which is associated with higher *SLC6A4* gene expression[233]- showed a superior reduction of depressive scores in response to SSRI treatment.

Box 2: Serotonergic system

Serotonin (5-HT) is synthesized from the amino acid L-tryptophan by the rate-limiting enzyme L-tryptophan-hydroxylase (TPH), resulting in the formation of 5-hydroxytryptophan (5-HTP)[234]. 5-HT is subsequently obtained by removal of a carboxyl group catalyzed by the enzyme 5-hydroxy-tryptophan-decarboxylase. 5-HT is, like other neurotransmitters, stored in storage and release vesicles via the vesicular monoamine transporter (VMAT). When released in the synapse, serotonin exerts its effects through 16 distinctive 5-HT receptors. Furthermore, 5-HT reuptake in the presynaptic neuron occurs via the serotonin transporter (5-HTT).

Serotonin synthesis

The efficacy of SSRIs is also influenced by polymorphisms in genes encoding proteins involved in 5-HT synthesis. For instance, a SNP (rs7163862, A:63%, T:37%, TT haplotype) in the promoter of the *GCHFR* (GTP-cyclohydrolase I feedback regulator) gene, regulating the 5-HT and dopamine synthesis cofactor tetrahydrobiopterin (BH4), reduced the responsiveness to fluoxetine[34]. Because the TT haplotype is associated with reduced gene transcription, this finding may imply that SSRI effects are diminished in individuals synthesizing relative low amounts of 5-HT. L-tryptophan hydroxylase 2 (TPH2) is the rate-limiting enzyme involved in 5-HT synthesis and exclusively found in the brain. Studies reported that the number of *TPH2*

rs2171363 (C:40%,T:60%) heterozygote carriers was higher in responders than non-responders to fluoxetine and citalopram[35], and that the *TPH2* C allelic variant of the rs10897346 (C>T) SNP, which is completely associated with functional Pro312Pro polymorphism affecting *TPH2* expression, was associated with an increased risk for non-responding to SSRIs[235]. However, another study did not observe an association between *TPH2* gene and SSRI efficacy[236] (Binder et al., 2004). Although it remains to be established how these polymorphisms affect *TPH2* gene transcription, the studies suggest that SSRI effects are dependent on brain 5-HT synthesis, and thereby 5-HT levels. The other TPH isoform, *TPH1*, mainly found in peripheral tissues, has not been associated with fluoxetine and venlafaxine treatment outcomes[237], although the response to citalopram[126] and paroxetine[178] treatment were worse in depressed patients carrying the *TPH1* A/A and A/C genotypes (rs1800532; c.218C>A, A:33%, C:67%) compared to those with the *TPH1* C/C genotype. These mixed findings for *TPH1* imply that particularly central 5-HT levels are relevant for SSRI effects.

Serotonin receptors

When 5-HT has been released in the synaptic cleft, its signal is transmitted to the genome through 5-HT receptors. In view of the finding that a progressive desensitization of serotonin 1A (5-HT_{1A}), and also 1B (5-HT_{1B}), autoreceptors enhances the action of SSRIs[238,239], the association between *HTR1A* (5-HT_{1A} receptor gene) and *HTR1B* (5-HT_{1B} receptor gene) polymorphisms and SSRI responses have been thoroughly investigated. Villafuerte and coworkers[127] analyzed the influence of functional polymorphisms in *HTR1A* (rs6295, rs1364043, rs1423691 and rs10042486) and *HTR1B* (rs6296, rs6297, rs6298, rs130058, rs11568817 and rs1221366) in depressed patients treated with citalopram, and found that depressed homozygotes for the G-allele at rs1364043 (G:32%, T:68%) in *HTR1A* and the C-allele at rs6298 (c.129C>T; C:71%, T:29%) in *HTR1B* showed a better response to citalopram over time. Furthermore, depressed homozygotes for the G-allele at rs6295 (-1019C/G; C:48%, G:52%) in *HTR1A* and at rs6296 in *HTR1B* showed significantly lower response rates[36-38]. It is possible that a higher baseline level of expression of these two receptors interferes with SSRI efficacy, as the *HTR1A* rs6295 G-variant prevents binding of a transcription repressor (NUDR/DEAF-1) in the raphe nuclei[240]. In addition, binding to HTR1B is increased in subjects homozygous for a G in the rs6296 (c.861G>C; C:36%, G:64%) polymorphism[241]. These findings raise the hypothesis that genetic variants enhancing 5-HT_{1A} and 5-HT_{1B} expression/function decrease desensitization of both receptors by SSRIs and ultimately drug responses. Reduced SSRI efficacy has also been noted in a population of anxiety patients, in which the GG *HTR1A* genotype of the rs6295 polymorphism was strongly associated with diminished changes in panic attack frequency following paroxetine or sertraline treatment[159]. However, also negative studies for *HTR1A* gene polymorphisms and their association with SSRI effects exist[242-245]. Also Arias and colleagues[246] did not find an association between the rs6295 polymorphism in the *HTR1A* gene and clinical outcome or remission after citalopram treatment. However, they found a combined genetic effect of HTR1A and SLC6A4 genes to influence the clinical outcome of depressive patients.

When considering the remission status, an increase of patients carrying the risk genotype combination (S/S-G/G) was found among subjects who did not reach remission.

Regarding HTR2A polymorphisms, rs7997012 and rs6311, in interaction with gender, explained 14% of chance in depression scores after SSRI treatment[39]. In another study, two functional HTR2A SNPs (-1438A/G: rs6311 and c.102C>T: rs6313) and two functionally undefined HTR2A SNPs (rs7997012 and rs1928040) were associated with the therapeutic response to SSRIs in Japanese MDD patients[40]. The association between the HTR2A rs7997012 polymorphism and the outcome of citalopram has also been shown by several others[128-130,174,175,220,246]. These findings may be consistent with reports that 5-HT2A receptor blockade leads to rapid antidepressant effects in rodents[247]. Notably, the pharmacogenetic effects of the c.102C>T *HTR2A* polymorphism may be dependent on ethnicity, as *HTR2A* T-allele carriers in a Japanese sample showed selective and slower score reductions than C-allele carriers in delusion and activity symptoms, whereas in an Italian sample C-allele carriers showed a slower and selective score reduction compared with T-allele carriers in somatic anxiety[175]. It may also be dependent on the interaction between the *HTR2A* gene and a new marker (rs1954787; C:50%, T:50%) in the *GRIK4* gene, which codes for the kainic acid-type glutamate receptor KA1. The effect size of the *GRIK4* marker alone was modest, but homozygous carriers of the treatment-response-associated marker alleles of both the *GRIK4* and *HTR2A* genes were 23% less likely to experience non responsiveness to treatment relative to participants who did not carry any of these marker alleles[249]. On the other hand, also negative findings regarding *HTR2A* gene polymorphisms and their association with SSRI effects exist. Illi and colleagues[244] could not find an association between an *HTR2A* polymorphism (rs6313) and major depressive disorder or SSRI response.

Other 5-HT receptor polymorphisms implicated in the pharmacogenetic effects of SSRIs are those located in *HTR2C* (5-HT_{2C} receptor gene), *HTR3A* (5-HT_{3A} receptor gene) and *HTR6* (5-HT_{6} receptor gene). In alcoholic patients, *HTR2C* Ser23 alleles were associated with significantly higher adrenocorticotropic hormone (ACTH) responses under placebo administration and these responses were reduced in the citalopram treated group[131]. Furthermore, nausea, a side effect of SSRI (paroxetine) treatment occurring in approximately 30% of patients, was significantly associated with an insertion/deletion polymorphism (-100_-102AAG) in the *HTR3B* receptor gene in a small cohort of 72 Japanese patients with anxiety or depression-related disorders. In other words, patients homozygous for the -100-102AAG insertion allele had a lower risk of developing nausea compared to other patients[176]. This finding may coincidence with the efficacy of 5-HT_{3} receptor antagonists in the prevention of chemotherapy induced nausea in cancer patients[250]. Finally, the association between SSRI efficacy and the *HTR6* gene has been studied, but none out of five *HTR6* polymorphisms (rs6693503, rs1805054, rs4912138, rs3790757 and rs9659997) were associated with SSRI responsiveness in a population of Japanese depressed patients[251].

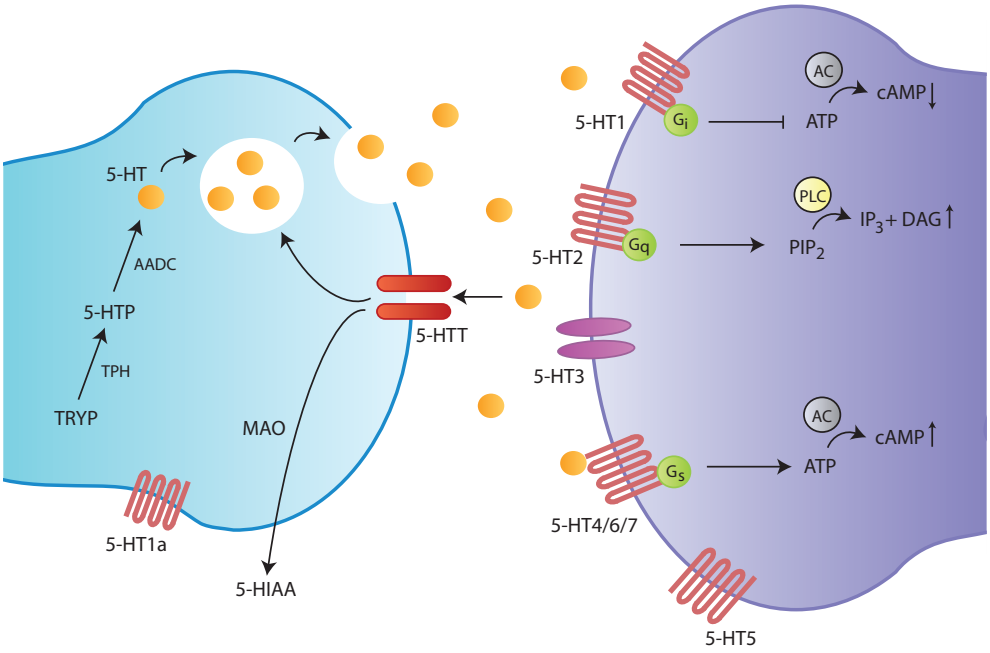

Figure 1. Schematic overview of the serotonergic system. Serotonin is synthesized by the conversion of tryptophan (TRYP) to 5-hydroxytryptophan (5-HTP) catalyzed by tryptophan hydroxylase (TPH), followed by conversion of 5-HTP to 5-hydroxytryptamine (5-HT, serotonin) catalyzed by aromatic aminoacid decarboxylase (AADC). 5-HT is taken up into storage vesicles and can be released from these vesicles into the synaptic space, where it can activate subtypes of the 5-HT receptor (1-7). Each can activate its own signal transduction pathway inside the postsynaptic neuron. 5-HT is taken up into the presynaptic neuron by the 5-HT transporter. Within the presynaptic neuron, 5-HT would either be taken up by the storage vesicles or degraded by monoamine oxidase (MAO). Selective serotonin-reuptake inhibitors (SSRIs) inhibit the 5-HT transporter. 5-HIAA, 5 hydroxyindolacetic acid; AC, adenylate cyclase; PLC, Phospholipase C; DAG, diacylglycerol; IP_3, inositol-1,4,5-trisphosphate; PIP_2, phosphatidylinositol-4,5-bisphosphate.

Animals-in vivo studies

The serotonin transporter

Surprisingly, whereas the 5-HTTLPR has been linked to SSRI efficacy in numerous human pharmacogenetic studies, rodent studies have reported conflicting findings on *Slc6a4* (encoding 5-HTT) expression: *Slc6a4* mRNA levels were either unaffected in the rat dorsal raphe nucleus (DRN) following 21 days of SSRI treatment[134,252-254], or 4-8 week SSRI treatment normalized increased stress-induced *Slc6a4* mRNA levels in the rat midbrain[43,44].

Serotonin transporter knockout animals

Given that the 5-HTTLPR S-allele in humans increases the vulnerability for anxiety and depression, 5-HTT knockout rodents are considered as valuable and reliable models for the 5-HTTLPR. As such, they show a disturbed serotonergic system. Extracellular 5-HT levels are

increased, and tissue 5-HT levels[255,256] and 5-HT_{1A} receptor sensitivity are reduced[257-261]. The 5-HTT knockout rodents also show increased anxiety by spending less time in the center of the open field[261-264], spending less time on the open arm in the elevated plus maze[261-263,265] and by showing increased latency to start eating in the novelty-suppressed feeding test[261,263,266]. Furthermore, 5-HTT knockout rodents show an increase in immobility in the forced swim test[261,262,266] and reduced sucrose preference[261,265], which are characteristics of behavioral despair and anhedonia, respectively. In addition, 5-HTT knockout rodents show impaired social behavior[256,264,267,268]. These depressive-like traits as a result of knock-down of the 5-HTT again pinpoint the importance of the serotonergic system in depression. However, the positive relationship between central 5-HT levels and depression in these animals undermines the monoamine hypothesis of depression.

Serotonin synthesis

Of interest, the *Tph2* gene, the rate-limiting enzyme involved in 5-HT synthesis in the brain, undergoes changes in expression in response to SSRIs. For instance, whereas chronic mild stress (CMS), a procedure to induce depression-like symptoms in rats[269] had no effect on *Tph2* mRNA levels, treatment for 4 weeks with citalopram (30 mg/kg/day) down-regulated *Tph2* mRNA levels in the DRN[134]. Likewise, Dygalo and colleagues[43] reported that chronic fluoxetine treatment (25 mg/kg/day per os (p.o.), 14 days) significantly reduced *Tph2* mRNA levels in the rat brainstem. However, long-term treatment of rats with another SSRI, sertraline (10 mg/kg, i.p., 2 weeks), was associated with an up-regulation of mRNA and protein TPH levels in the DRN, as determined by *in situ* hybridization and immunocytochemistry, respectively[160]. Furthermore, Heydendael and Jacobson[45] showed an increase in DRN *Tph2* gene expression in mice after fluoxetine treatment (5 mg/kg, i.p.) for 5 weeks. The discrepancies between studies showing decreased and increased *Tph2* mRNA levels in response to chronic SSRI treatment may be due to differences in factors like SSRI type, treatment duration, route of administration, rat strain, and/or mRNA detection method. Of interest, Kim and colleagues[160] showed that the up-regulation of *Tph* gene expression in the DRN by sertraline was attenuated by the protein kinase A (PKA) inhibitor N-[2-(p-bromocinnamylamine)-ethyl]-5-isoquinolinesulfonamine, suggesting that a mechanism involving the PKA signaling pathway might at least in part mediate the therapeutic action of sertraline. This finding matches reports that long-term antidepressant administration leads to PKA nuclear translocation[270] and increased expression of cAMP-response element-binding protein (CREB)[55]. The human *TPH* gene contains a cAMP-responsive element in its promoter[271], and its gene expression[272-274] is up-regulated by cAMP, which may also be the case in rodents.

Serotonin receptors and signaling pathways

Regarding the involvement of 5-HT receptors in SSRI-mediated molecular mechanisms in experimental animals, it was found that 24 h after a 2-3 week treatment with fluoxetine (8 mg/kg i.p., daily) there was a significant decrease in *Htr1a* mRNA levels in the anterior raphe

area (comprising the median and dorsal raphe nuclei) and a significant increase in *Htr1b* mRNA levels in the striatum and the cerebral cortex. Electrophysiological recordings in brain slices showed that chronic fluoxetine treatment reduced the potency of the 5-HT_{1A} agonist 8-hydroxy-2-(di-n-propylamino)tetralin to inhibit neuronal activity in the DRN, but did not affect 5-HT_{1A}-evoked responses of CA1 pyramidal cells in the hippocampus. The decrease in *Htr1a* mRNA levels in the anterior raphe suggests that fluoxetine-induced desensitization of 5-HT_{1A} autoreceptors involves changes at the transcription level[14]. The regulation of 5-HT_{1B} receptor transcripts by SSRI treatment was also addressed in another rat study. It was found that chronic (8 weeks) fluoxetine (5 mg/kg/d) and paroxetine (5 mg/kg/d) administration to rats via subcutaneous osmotic minipumps reduced DRN *Htr1b* mRNA by 36% and 27%, respectively, whereas sertraline (10 mg/kg/d) had a no significant effect. After 3-14 days of drug washout, DRN *Htr1b* mRNA levels in SSRI treated rats were no longer different from controls. *Htr1b* mRNA levels in hippocampus were not affected by SSRI drugs at any time point. These results confirm that SSRI antidepressants selectively reduce presynaptic *Htr1b* mRNA levels, and that this effect is maintained for at least 8 weeks of antidepressant treatment, but reverses rapidly after discontinuation[46]. Chronic SSRI (fluoxetine) treatment has also been reported to down-regulate *Htr2c* mRNA expression in the mouse prefrontal cortex, which became significant after 3 weeks of treatment and persisted after a fourth week of drug withdrawal. In the hippocampus, fluoxetine induced a threefold up-regulation of *Htr2c* mRNA levels by the 2nd and 3rd week, returning to the baseline level after drug withdrawal. These robust changes in *Htr2c* mRNA levels were associated with slight changes in *Htr2c* RNA editing[47]. *Htr2c* receptor mRNA editing may be strain specific, as only BALB/c, but not C57BL/6 and 129Sv, mice showed significant site-specific increases in *Htr2c* pre-mRNA editing, leading to an increase in the pool of mRNA encoding receptors with reduced function in response to chronic fluoxetine treatment. Chronic stress exposure blocked this effect of fluoxetine, suggesting that *Htr2c* receptor mRNA editing is involved in the chronic effects of fluoxetine[275]. Overall, the 5-HT_{2C} findings are consistent with the view that fluoxetine acts as a competitive and reversible antagonist of 5-HT_{2C} receptors[48]. Interestingly, there is a physical interaction between PTEN - a negative regulator of the PI3-kinase pathway converging on molecules capable of influencing morphogenesis, growth, and neuronal function, including mTOR, GSK-3beta, CREB, and NF-kappa-B- and the 5-HT_{2C} receptor: the phosphatase activity of PTEN regulates the activity of this receptor[276]. Hence, it is possible that alterations in *Htr2c* mRNA levels induced by chronic SSRI treatment are associated with changes in a cascade of intracellular signaling components regulating the expression of genes involved in neuronal morphogenesis.

5-HT receptors targeted by SSRIs are linked to cAMP-PKA and PLC-PKC pathways converging on CREB. As such, 5-HT_{2C} receptors stimulate, through Gαq, phospholipase C (PLC) activity, which subsequently promotes the release of diacylglycerol and inositol triphosphate, which in turn stimulate protein kinase C (PKC) activity and Ca^{2+} release. In this context, a study assessing whole brain kinase expression using Affymetrix gene chips in rats treated

with placebo 3 and 21 days, fluoxetine 3 and 21 days, and citalopram 21 days revealed that PKC-delta, PKC-gamma, stress-activated protein kinase, cAMP-dependent protein kinase beta isoform, Janus protein kinase, and phosphofructokinase M were all down-regulated[50]. These findings fit well with the studies above demonstrating *Htr2c* down-regulation in response to chronic SSRI treatment. Furthermore, long-term citalopram (10 mg/kg, i.p.), but not single-dose administration, increased the adenylyl cyclase (AC) type 1 mRNA in the hippocampus, whereas type 2 mRNA was unaffected. However, long-term citalopram treatment did not increase AC type 1 protein, basal or forskolin-stimulated AC activity, but GTP increased AC activity in the hippocampus. This may indicate enhanced AC/G protein coupling. Thus, citalopram may increase cAMP signaling by acting on components of the AC system without affecting the protein level of the AC type 1[136]. A role for cAMP signaling is further supported by the finding that rolipram, a selective inhibitor of cAMP-specific phosphodiesterases type 4 (PDE4), has antidepressant efficacy in behavioral models of depression and in clinical trials. PDE4 metabolizes cAMP[277], and by inhibiting cAMP breakdown PDE4 inhibitors can potentiate signaling through the cAMP system. *In situ* hybridization analysis indicated that chronic fluoxetine treatment (5 mg/kg, i.p., 21 days) significantly increased *Pde4a1*, but not *Pde4a5* or *Pde4a10*, mRNA levels in frontal and parietal cortices. Fluoxetine also increased *Pde4a10* levels in the hippocampus. The differential up-regulation of *Pde4a* splice variants suggests compensatory region-specific responses to the antidepressant-induced increase in cAMP signaling[51]. Another study analyzing the mRNAs coding for PDE4A, PDE4B, PDE4D, and the five known PDE4D splice variants using *in situ* hybridization in response to acute (1 day) and chronic (14 days) fluoxetine (3 mg/kg day) treatment in rats revealed that single fluoxetine administration increased the density of *Pde4d1* mRNA in frontal and hippocampal regions. Chronic fluoxetine treatment increased *Pde4a* mRNA levels in the hippocampus and decreased *Pde4a* levels in the anterior cingulate cortex. Furthermore, chronic fluoxetine was associated with mostly increased *Pde4d(5*) mRNAs in areas including the subiculum, dorsal part of medial geniculate nucleus, parabigeminal nucleus, pontine nuclei, vestibular nuclei, area postrema, and cerebellum[52,53]. Takahashi and coworkers[52] showed that the expression of PDE4A and PDE4B, but not PDE4D, mRNA and immunoreactivity were significantly increased in rat frontal cortex by 14-day administration of sertraline (10 mg/kg, i.p.) and fluoxetine (5 mg/kg, i.p.). They also found that antidepressant administration significantly increased the expression of *Pde4b* mRNA in the nucleus accumbens, a brain region thought to mediate pleasure and reward that could also contribute to the anhedonia often observed in depressed patients. In contrast, expression of *Pde4a* and *Pde4b* were not influenced by short-term treatment (1 or 7 days). The changes in cAMP-specific phosphodiesterases may be linked to CREB, a well-known transcription factor linked to G-protein coupled receptors and cAMP signaling. Specifically, chronic administration of the PDE inhibitors rolipram or papaverine increased expression of *Creb* mRNA in hippocampus, an effect also found following chronic SSRI treatment. These effects were associated with increased levels of CRE immunoreactivity and of CRE binding activity. Increased expression and function of CREB

suggest that specific target genes are regulated by sertraline (10 mg/kg, i.p.) or fluoxetine (5 mg/kg, i.p.) treatment[55].

In further support of the findings that SSRIs affect gene transcription via CRE, it was found that chronic (14 days), but not acute, fluoxetine (10 mg/kg, i.p.) administration to transgenic mice with a CRE-LacZ reporter gene construct significantly increased CRE-mediated gene transcription, as well as the phosphorylation of CREB in the cerebral cortex, hippocampus, amygdala, and hypothalamus[278]. Whereas these findings overall show that SSRI treatment leads to up-regulation of the cAMP-CREB pathway (with increased PDE4 levels being indicative of higher levels of cAMP available for breakdown), it has been reported that citalopram (25 mg/kg, i.p.) and fluoxetine (10 mg/kg, i.p.) treatment (21-25 days) completely abolished the increase in CRE/CREB-directed transcription in the hippocampus induced by chronic psychosocial stress[56]. A similar finding was obtained by Abumaria and coworkers[134]. This finding contributes to the debate whether CREB is antidepressive, or pro-depressive[279].

Serotonin receptors and signaling pathways in knockout animals

Knockout rodents have further increased insights in the molecular targets of SSRIs: like polymorphisms in humans, genetic background of rodents influences the efficacy and side effects of SSRIs. As such, it has been reported that citalopram induced a dose-dependent inhibition of rapid eye movement (REM) sleep 2-6 h after injection, which was observed in wild-type and 5-$HT_{1B}^{-/-}$ mice, but not in 5-$HT_{1A}^{-/-}$ mutants. This REM sleep inhibition was fully antagonized by pretreatment with the 5-HT_{1A} antagonist WAY 100635, but only partially with the 5-HT_{1B} antagonist GR 127935, indicating that the action of the SSRI citalopram on sleep in the mouse is essentially mediated by 5-HT_{1A} receptors[135]. Another study showed that a mouse model bearing a loss of function mutation in G(alpha)i2 (G184S), a regulator of G protein signaling (RGS) coupled to 5-HT_{1A} receptors, exhibited spontaneous antidepressant- and anxiolytic-like behavior, as well as increased cortical and hippocampal phosphorylation of glycogen synthase kinase-3beta, a constitutively active pro-apoptotic kinase that is inhibited through phosphorylation in response to 5-HT, SSRIs, and 5-HT_1 receptor agonists. These mice were 5-10 times more responsive to the antidepressant-like effects of the SSRI fluvoxamine (30 mg/kg i.p.), suggesting that endogenous RGS proteins suppress the antidepressant-like effects of SSRIs[211]. In 14-day old 5-HT_{2C} receptor mutant mice with a heterozygous mutation of the beta-endorphin gene (2CREnd mice) acute fluvoxamine (10 and 30 mg/kg) administration exerted anorexic effects, whereas fluvoxamine had no effect on food intake in age-matched wild-type mice and 5-HT_{2C} receptor mutant mice. These results suggest that acute fluvoxamine-induced feeding suppression requires a concerted perturbation of the 5-HT_{2C} receptor and beta-endorphin signalling[210]. In another study, Nonogaki and colleagues[280] showed that fluvoxamine together with pharmacological inactivation of 5-HT_{2C} receptors (without mutation of the beta-endorphin gene) exerts appetite suppression and that these effects were attenuated in the presence of a selective 5-HT_{1B} receptor antagonist, suggesting that activation of 5-HT_{1B} receptors and blockage of 5-HT_{2C} contribute to the

appetite-suppressing effects. Finally, it has been reported that acute administration of three SSRIs (citalopram (3 mg/kg, s.c.), fluoxetine (5 mg/kg, s.c.), paroxetine (1 mg/kg, s.c.)), but not the tricyclic antidepressant desipramine (2 mg/kg, s.c.), had a significantly stronger REM sleep suppressive effect in 5-$HT_7^{-/-}$ mice compared to 5-$HT_7^{+/+}$ mice. In contrast, REM sleep was similarly affected in 5-$HT_7^{+/+}$ mice and 5-$HT_7^{-/-}$ mice after treatment with the 5-HT_{1A} receptor agonist ipsapirone and the 5-HT_{1A} receptor antagonist WAY-100635. These findings indicate that the 5-HT_7 receptor augments the effect of various acutely administered SSRIs on REM sleep suppression and that this effect is distinct from those mediated via 5-HT_{1A} receptors[49].

Animals-in vitro studies

The serotonin transporter and serotonin synthesis

Animal *in vitro* studies addressing the effects of SSRIs on the serotonergic system are limited. An expression microarray analysis of citalopram-treated serotonergic neurons revealed that antidepressant treatment did not alter Slc6a4 mRNA expression, whereas evidence by confocal microscopy was obtained for citalopram-induced 5-HTT internalization. These data suggest that 5-HTT trafficking from and to the cell membrane is regulated at the post-transcriptional level[281], a finding also reported by Iceta and coworkers[282] and matching in vivo rodent findings. Another study using RBL-2H3 cells showed an increase in mRNA and protein levels of TPH by sertraline, as determined by Northern blot and immunoblot analyses, respectively. This was accompanied by increases in the levels of TPH enzymatic activity and total 5-HT levels[160]. This increase in TPH level by SSRI treatment corresponds well with part of the in vivo studies. However, as mentioned above, there are also in vivo studies showing a decrease in TPH expression.

Dopamine, norepinephrine, GABA, glycine and glutamate system

5-HT does not function in isolation, but interacts with many other neurotransmitter systems, including the dopamine (DA), norepinephrine (NE), GABA, glycine and glutamate systems (Box 3). Microdialysis studies *in vivo* have clearly shown that exposure of the striatum or nucleus accumbens to 5-HT results in increased release of DA[283,284]. The NE system is activated after administration of 5-HT receptor antagonists in the locus coeruleus of rats, showing an interaction between the 5-HT and NE pathways[285]. Allain and colleagues[286] showed that 5-HT interacts with the gamma-aminobutyric acid (GABA) system, by controlling the maturation of the GABA phenotype in the ventral spinal cord via 5-HT_{1B} receptors. A modulatory effect of 5-HT on the glycine response was shown in the rat sacral dorsal commissural nucleus by Xu *et al.*[287]. Furthermore, an interaction between glutamate receptor mGLU5 and the 5-HT_{2A} receptor was shown in mice[288].

Box 3: Dopamine, norepinephrine, GABA, glycine and glutamate system
Dopamine (DA) is a neurotransmitter synthesized from tyrosine via tyrosine hydroxylase (TH) catalysis to levodopa (L-DOPA), and subsequent decarboxylation by dopa decarboxylase (DDC) to DA. It can activate five known types of DA receptors (D1, D2, D3, D4, and D5), which downstream pathways are involved in many processes, like motivation, sleep, mood and learning. DA is de-activated by reuptake by the presynaptic neuron and is broken down by the enzymes catechol-O-methyl transferase (COMT) and monoamine oxidase (MAO). Norepinephrine (NE) is a neurotransmitter synthesized from DA by DA β-hydroxylase and released from noradrenergic neurons in the locus coeruleus. The actions of NE are carried out via the binding to adrenergic receptors (α_1, α_2, β_1, β_2 and β_3). Glutamate is the most abundant excitatory neurotransmitter, which plays a role in synaptic plasticity. Glutamate binds to α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPARs), metabotropic glutamate receptors (mGluRs), kainate receptors and NMDA receptors (NMDARs) activating downstream pathways, like the BDNF pathway (see Box 4) and the calcium signaling pathway[312]. Glutamate also serves as the precursor for the synthesis of the inhibitory gamma-aminobutyric acid (GABA) in GABA-ergic neurons, which is catalyzed by glutamate decarboxylase (GAD). GABA acts at inhibitory synapses by binding to specific transmembrane receptors, $GABA_A$ and $GABA_B$, and plays a role in regulating neuronal excitability throughout the nervous system and muscle tone. Glycine is a neurotransmitter which can exert both inhibitory and excitatory functions. When glycine receptors are activated, chloride ions enter the neuron and the cell undergoes a hyperpolarization, but glycine is also involved in the activation of NMDA receptors as a co-agonist of glutamate.

Human-polymorphisms
In line with evidence that the serotonergic and dopaminergic systems interact[289], it has been found that the Met allele of the Val(108/158)Met polymorphism (rs4680) in the catechol-O-methyltransferase (*COMT*) gene was associated with a faster onset of therapeutic effects of fluvoxamine and paroxetine in a population of depressed patients[212,290]. The *COMT* Val(108/158)Met polymorphism was not associated with a 4-week fluoxetine therapeutic response, but association analysis showed that patients with the COMT(Val/Val) genotype had poorer responses in the eighth week[291]. However, also negative findings for COMT Val(108/158)Met gene variance have been obtained[292-294]. Another SNP (rs13306278; C:92%, T: 8%), located in the distal promoter region of *COMT*, showed significant association with remission in depressed patients treated with citalopram or escitalopram. Alternation in the ability of the variant sequence to bind nuclear proteins might affect transcription regulation[295]. Others showed that the dopamine transporter (DAT) VNTR polymorphism can influence rapid response to antidepressant therapy. There was a significantly smaller number of rapid responders among homozygous carriers of the DAT1 9-repeat allele (9/9) than among heterozygous (9/10) and homozygous (10/10) carriers of the 10-repeat allele[296]. Polymorphisms in the dopamine D_2 and D_4 receptors were not associated with SSRI efficacy[297].

Regarding the glycine system, a metabolic assay of plasma samples from escitalopram remitters and nonremitters showed that glycine, as an inhibitory neurotransmitter, was

negatively associated with treatment outcome, an effect that was associated with the rs10975641 SNP in the glycine dehydrogenase gene[139]. Given that glycine/N-Methyl-D-aspartic acid (NMDA) receptor functional antagonists enhance the antidepressant-like action of 5-HT[298], and that oral glycine administration enhances 5-HT release in the prefrontal cortex[299], it may be that 5-HT and glycine synergistically mediate the (neuroplastic) effects of SSRIs.

No association could be found between purinergic receptor P2X, ligand-gated ion channel 7 (*P2RX7*) gene polymorphisms and MDD or remission after SSRI treatment[300], whereas these polymorphisms have been suggested to be associated with MDD[301]. P2RX7 is an ATP-gated non-selective cation channel activated by high concentrations of ATP (>100 μM)[302]. P2RX7 is localized on glutamatergic nerve terminals and plays a significant role in ATP-evoked glutamate efflux[303]. Furthermore, P2RX7 mediates the influx of Ca^{2+} and Na^{+} ions and the release of proinflammatory cytokines**.** However, a polymorphism in another glutamatergic gene, *GRIK4*, in combination with an *HTR2A* polymorphism is associated with SSRI response[249]. The rs5443 (c.825C>T; C:52%, T:48%) polymorphism in the G-protein beta3 subunit (*GNB3*) gene - a key modulator of signal transduction – shown to be involved in dopamine receptor availability- is associated with SSRI responsiveness[304]. Lin and colleagues[245] showed that carriers of the C-allele responded better to SSRI treatment than those with the T/T variant. Conversely, others showed that subjects with *GNB3* T/T variants showed better response to fluoxetine[305], fluvoxamine and paroxetine treatment[209]. In addition, there are also studies showing no association between the rs5443 polymorphism and SSRI response[306].

Yet, glycogen synthase kinase-3beta (*GSK3B*) TAGT carriers showed a poorer response to chronic fluoxetine and citalopram treatment[61]. GSK3B is a constitutively active serine/threonine kinase that phosphorylates cellular substrates and thereby regulates a wide variety of cellular functions, including development, metabolism, gene transcription, protein translation, cytoskeletal organization, cell cycle regulation, and apoptosis[307]. GSK3B is regulated by several neurotransmitter systems, including serotonergic[308], dopaminergic[309], cholinergic and glutamatergic systems[310]. No correlation between the norepinephrine transporter T182C/G1287A polymorphisms and SSRI response was observed[30,33].

There are also studies showing evidence for the involvement of the glutamate system on sexual dysfunction, a well-known side effect of SSRI treatment. For instance, sexual dysfunction as side effect of citalopram treated in a cohort of Caucasian depressed patients was associated with single nucleotide polymorphisms in ionotrophic α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) glutamatergic receptor genes: *GRIA3* (ionotrophic AMPA 3) and *GRIK2* (ionotropic kainate 2) were associated with decreased libido, *GRIA1* (ionotrophic AMPA 1) was associated with difficulty achieving orgasm, and *GRIN3A* (ionotropic N-methyl-D-aspartate 3A) was associated with difficulty achieving erection. Surprisingly, no associations between sexual dysfunctions and serotonergic genes were found[138]. Furthermore, Thomas and colleagues[60] showed evidence for an association between the adrenergic beta-1 receptor gene (*ADRB1*) Ser49Gly (rs1801252; A:85%, G:15%)

and Arg389Gly (rs1801253; C:69%, G:31%) variants and cardiovascular side effects. There was a significant interaction between Arg389Gly variant status and fluoxetine and paroxetine treatment in relation to systolic blood pressure and heart rate.

Human-lymphoblasts

A study in human lymphoblasts focused on G protein-coupled receptor kinases (GRKs). Blood platelets derived from unmedicated depressed patients were characterized by reduced *GRK2* mRNA levels and chronic SSRI treatment normalized platelet *GRK2*. Furthermore, *GRK2* up-regulation discriminated between responder and non-responder patients[59]. GRK2 is a ubiquitous protein kinase of the GRK family, which phosphorylates the beta adrenergic receptor and related G-protein-coupled receptors[311]. A study in human mononuclear leukocytes also showed alleviation of decreased *GRK2* mRNA levels by antidepressant treatment[137].

Animals-in vivo

Besides studies in humans, the effect of SSRIs on other neurotransmitter systems has also been investigated in animals *in vivo*. In rats, chronic (21 days) fluoxetine treatment failed to affect dopamine D_3 receptor mRNA levels on the nucleus accumbens shell, although an up-regulation was seen after 42 days of treatment[63]. In addition, repeated administration of citalopram (10 mg/kg, p.o., 14 days) resulted in an increase in the level of D_1 (*Drd1*) mRNA in the striatum and in the core region of nucleus accumbens[140]. Furthermore, mRNA levels of tyrosine hydroxylase (*Th*), the enzyme mediating DA synthesis, were increased by 70-150% in the locus coeruleus after 2 weeks of fluoxetine treatment, and by 71-115% after 8 weeks of treatment[64]. An increase in *Th* mRNA levels after fluoxetine treatment (5 weeks, 5 mg/kg) was also reported by Heydendael and Jacobson[45]. Thus, overall, chronic SSRI treatment may lead to up-regulation of DA neurotransmission. Given that NE is synthesized from DA, it is expected that SSRIs affect the expression of noradrenergic genes as well. However, citalopram (10 mg/kg, i.p., 14 days) failed to affect the expression of alpha1A and alpha1B subtypes of the adrenergic alpha1-A receptor in the rat prefrontal cortex, as measured by Northern blot analysis and RT-PCR, respectively[313].

Knockout animals

Yet, mice lacking the dopamine beta-hydroxylase gene ($Dbh^{-/-}$), converting DA into NE, did not respond to the antidepressant effects of fluoxetine, sertraline, and paroxetine in the tail suspension test, whereas citalopram was equally effective at reducing immobility in mice with and without NE. Restoration of NE using L-threo-3,4-dihydroxyphenylserine reinstated the behavioral effects of paroxetine in $Dbh^{-/-}$ mice, thus demonstrating that the reduced sensitivity to antidepressants is related to NE function[68]. In mice lacking GABA Type A chronic but not subchronic treatment with fluoxetine (~4 mg/kg/day in drinking water) normalized anxiety-like behavior in the novelty suppressed feeding test, but not depression-related

behavior in the forced swim, tail suspension, and sucrose consumption tests[69]. This reduced sensitivity to fluoxetine in the GABA Type A knock out indicates that GABA is important for the anti-depressive effects of fluoxetine.

Animals-in vitro

Data on the monoamine and excitatory/inhibitory neurotransmitter systems interacting with SSRIs are limited for *in vitro* animal studies. Yet, interesting is the finding that in cultured mouse astrocytes fluoxetine up-regulated the kainate receptor *Grik2* mRNA levels and its RNA editing. This effect was associated with abolishment of glutamate-mediated increases in intracellular Ca(2+) and ERK(1/2) phosphorylation, due to the 5-HT_{2B} receptor agonism of fluoxetine, as assessed by small interfering RNA manipulation[71]. This suggests that the fluoxetine-induced effects on the glutamatergic system are mediated via the HT_{2B} receptor.

Neuroplasticity factors

The serotonergic system also strongly interacts with brain-derived neurotrophic factor (BDNF), in a synergic manner[314]. BDNF is one of the targets, but also regulators, of the cAMP-CREB pathway, as well as a key factor in neuroplasticity and neurogenesis (Box 4). In addition, we will discuss some other neuroplasticity factors that might be influenced by SSRIs, like vascular endothelial growth factor (VEGF), dysbindin, synaptophysin, growth-associated protein-43 (Gap-43) and the Kv4.2 gene. VEGF participates in physiological and pathological processes of angiogenesis and studies of recent years have revealed neurotrophic and neuroprotective potential of VEGF in the nervous system. Furthermore, VEGF might play a role in the strong positive correlation between the incidence of cardiovascular diseases and depression[315]. Dysbindin is a protein that has a possible biological role in synaptic signaling and plasticity, and localization in the brain. The dysbindin complex is found in axon bundles and is a component of the dystrophin glycoprotein complex involved in glutamatergic neurotransmission[316], which may play an important role in the antidepressant potential of SSRIs. Synaptophysin is a synaptic vesicle glycoprotein which plays an essential role in synaptic plasticity[317]. Gap-43 is a growth and plasticity factor which is expressed at high levels in neuronal growth cones during development and axonal regeneration and is phosphorylated after long-term potentiation and after learning[318,319]. The Kv4.2 gene (*KCND2*) encodes for an essential subunit of voltage-gated A-type potassium channels that is involved in dendritic signal integration and synaptic plasticity[320].

Human-polymorphisms

The human *BDNF* gene bears the common Val66Met (rs6265; A:18%, G:82%) polymorphism, in which G>A substitution in nucleotide position 196 results in a valine (Val) to methionine (Met) change at amino acid position 66, with BDNF secretion being reduced in 66Met BDNF neurons[322]. A significant association of Val/Met heterozygous genotype and increased SSRI

response rate was found in several studies[323-325]. However, Chi and colleagues[326] reported that patients with the Val/Val genotype had significantly higher chances to be responders to venlafaxine treatment (serotonin-norepinephrine reuptake inhibitor) and that fluoxetine treatment was not associated with the Val66Met polymorphism. Furthermore, a study in Korean MDD patients showed that the Val66Met polymorphism is associated with citalopram efficacy[143], with Met-allele carriers responding better to citalopram treatment. In addition, Met-allele carriers showed a lower incidence of side effects including insomnia and decreased libido[77]. Moreover, there are studies showing no association between SSRI treatment and the Val66Met polymorphism[325]. The inconsistencies in results may be explained, at least partially, by ethnicity[327] and differences between antidepressants. Most studies that revealed significant association between the Val66Met polymorphism and antidepressants treatment response were based on Asian populations, whereas Caucasian studies showed no association. Another study showed that two *BDNF* polymorphisms (rs908867 and rs1491850) with unknown functions were associated with SSRI treatment outcome in obsessive compulsive disorder patients[78]. In depressed patients receiving SSRI treatment the *BDNF* rs962369 polymorphism was associated with increased suicidal ideation, by interacting with variants in *BDNF* and *NTRK2*, the gene encoding the BNDF receptor[196]. A haplotype analysis further revealed that p75(NTR) TCT carriers had a more unfavorable response to SSRI therapy[79]. Of interest, the rs2227684-G (A:43%, G:57%) and rs7242-T (G:43%, T:57%) alleles of the plasminogen activator (tPA) inhibitor type 1 (*PAI-1*) gene were found to be associated with depression in Chinese patients, and the haplotype derived from the rs2227631-G (A:41%, G:59%) and rs1799889-4G (-:35%, G:65) polymorphisms was lower in responders than non-responders to fluoxetine or citalopram treatment[80]. The activity of the tPA–plasminogen system is dependent on the generation of a protease, plasmin, which is generated from an inactive precursor, plasminogen, through the action of tPA. Plasmin is the most significant protease involved in the cleavage of proBDNF in the brain[328], and because proBDNF has biologically opposite effects to BDNF[329], the proteolytic processing of proBDNF by the tPA–plasminogen system thus represents a powerful means of controlling the direction of BDNF action. Therefore, the finding that both *BDNF* and *PAI-1* genes influence the efficacy of SSRIs confirms that these factors are functioning in a pathway. Taken together, these findings support the interaction between the serotonergic and BDNF-tPA systems.

This interaction might be quite specific, as no association between SSRI efficacy and vascular endothelial growth factor (*VEGF*) was found in Japanse depressed patients[330]. However, there are studies showing interactions between SSRIs and other neuroplasticity factors. The dysbindin gene (dystrobrevin-binding-protein 1, *DTNBP1*) SNP rs760761 showed a trend of difference between responders and non-responders (4th week) to SSRI treatment in depressed patients[144].

Human-lymphoblasts

In a study in which cultured human neuroblastoma SH-SY5Y cells were treated with fluoxetine

(as well as other classes of antidepressant drugs) for 6, 24 and 48 hours, no changes in *BDNF* mRNA levels were noted[331]. Furthermore, one study employed a genome-wide profiling approach (microarrays) in human lymphoblastoid cell lines (LCLs), and it was found that increased paroxetine LCL growth inhibition was associated with a 36-fold down-regulation of *CHL1*, a gene coding for a neuronal cell adhesion protein implicated in correct thalamocortical circuitry, schizophrenia and autism. Several additional genes implicated in synaptogenesis and/or in psychiatric disorders, including *ARRB1* (arrestin, β1, G-protein-coupled receptor-mediated synaptic transmission), *CCL5* (chemokine [C–C motif] ligand 5, chemoattractant for blood cells), *ENDOD1* (endonuclease domain containing 1, Associated with CNS myelin), *ENPP2* (ectonucleotide pyrophosphatase/ phosphodiesterase 2, Regulates myelin formation), *FLT1* (FMS-related tyrosine kinase 1, receptor implicated in synaptic transmission), *GABRA4* (gamma-aminobutyric acid receptor A, involved in GABAergic neurotransmission), *GAP43* (growth-associated protein 43, Regulates neurite outgrowth and plasticity), and *SPRY2* (Regulates FGF2 signaling), also displayed more than 1.5-fold lower basal expression in the paroxetine-sensitive LCLs[181]. Thus, it appears that the SSRI paroxetine particularly affects the expression of plasticity-associated genes, whereas 5-HT related genes (*SLC6A4*, 5-HT receptor genes and other candidate genes related to 5-HT signaling[221]) mostly addressed in pharmacogenetic studies are not affected. Notably, although lymphocytes and other human cell lines are thought to potentially act as a neural probe for studying psychiatric disorders[332], these findings should be considered with caution given that gene expression and regulatory mechanisms can be highly tissue specific.

Box 4: BDNF

Brain-derived neurotrophic factor (BDNF) is synthesized through proteolytic cleavage of proBDNF by the tPA/plasmin cascade. ProBDNF and mature BDNF (mBDNF) have been shown to elicit opposite effects by activating two distinct receptor-signaling systems. The biological function of BDNF is mediated by the TrkB receptor. Upon binding, BDNF triggers TrkB dimerization followed by autophosphorylation and the recruitment of a series of intracellular proteins that primes subsequent activation of several signaling pathways. In contrast, proBDNF binds p75NTR, resulting in the activation of several signaling molecules, including NF-kB, JNK and RhoA. The outcomes are opposite to those mediated by BDNF-TrkB. Antidepressant drugs are thought to increase BDNF levels, and thereby to increase neurogenesis and other pro-neuronal functions [321].

Animals-in vivo

To assess the time-dependent effects of SSRIs on *Bdnf* gene expression, rats were treated with fluoxetine (10 mg/kg, p.o.) for 4, 7, 14 and 21 days. Four days of fluoxetine treatment induced decreases in *Bdnf* mRNA (hippocampus, medial habenular and paraventricular thalamic nuclei), 7 days of treatment showed a non-significant increase in *Bdnf* mRNA, and there were marked and region-specific increases following 14 days of treatment. BDNF protein levels remained unaltered until 21 days of fluoxetine treatment, when the numbers of BDNF

immunoreactive cells were increased, reaching significance in the pyramidal cell layer of CA1 and CA3 regions of Ammon's horn, but not in the other sub-regions of the hippocampus. In contrast to the time dependent and biphasic response of the *Bdnf* gene to fluoxetine, activity-regulated, cytoskeletal-associated protein (*Arc*) mRNA showed a gradual increase during the 14-day course of treatment[81]. Also citalopram (10 mg/kg) treatment led to increases only: after short-term (2 days) treatment *Bdnf* mRNA was increased in the CA2 region of the hippocampus, and when combined with exercise, *Bdnf* mRNA levels increased in the CA4 and dentate gyrus after 2 weeks of treatment[145]. Again another study revealed a reversed biphasic BDNF response. Seven days of treatment with the SSRI escitalopram (10 mg/kg, i.p.) increased mRNA levels (as measured using the RNase protection assay) of BDNF in the rat prefrontal cortex, which was confirmed at protein level and associated with increased protein levels of phospho-ERK1/2 and p38 MAPK, signaling components linked to BDNF. Moreover, *Carf* (calcium responsive transcription factor) mRNA levels, a transcription factor of activity-dependent BDNF expression, were increased, possibly through activation of p38 MAPK and ERK1/2. At 21 days of treatment with escitalopram, *Bdnf* and *Carf* mRNA, and phospho CREB levels were decreased in the hippocampus, while phospho-p38 MAPK levels were increased in the hippocampus[195]. SSRI effects on *Bdnf* mRNA expression are thus region-specific, although some studies failed to show any changes in *Bdnf* mRNA levels in the dentate gyrus upon chronic fluoxetine treatment[86,333,334]. SSRIs also affect other neuroplasticity factors in rodents. As such, a transient increase in synaptophysin mRNA was observed in the hippocampus, and in the CA3 region a reduction of *Gap43* mRNA was observed after fluoxetine treatment (10 mg/kg, i.p.) for 7 and 14 days[86]. Overall it appears that SSRIs increase mRNA levels of *Bdnf* and synaptophysin, which most likely allow beneficial neuroplastic changes in, among others, the hippocampus.

Knockout animals

In $BDNF^{+/-}$ mice the SSRI paroxetine (8 mg/kg, i.p., acute) failed to increase hippocampal extracellular 5-HT levels, while it produced robust effects in wild-type littermates, suggesting that $BDNF^{+/-}$ mice show genetic resistance to serotonergic antidepressant drugs[183] and that 5-HT and BDNF interact synergistically. In support, fluvoxamine (50 and 100 pmol) significantly decreased 5-HT clearance in hippocampal slices of $BDNF^{+/+}$ mice, but not in $BDNF^{+/-}$ mice[183,213]. Furthermore, mice carrying a knockout of the Kv4.2 gene (*Kcnd2*)- which codes for an essential subunit of voltage-gated A-type potassium channels – were resistant to the antidepressive effects of acute fluoxetine (30 mg/kg, i.p.) in the forced swim and tail suspension test[87].

Hypothalamic system

It has also been well established that depression is associated with alterations in the hypothalamus-pituitary-adrenal (HPA)-axis (Box 5). Besides the HPA axis, other hypothalamic processes like regulation of hunger and reproduction are also associated with depression and

antidepressants.

Human-polymorphisms

Studies investigating the association between SSRI treatment and SNPs in genes involved in the HPA-axis are rather limited. The rs242941 (G:68%, T:32%) G/G genotype of the corticotrophin-releasing hormone receptor 1 (*CRHR1*) gene was associated with fluoxetine's therapeutic response in depressed patients characterized by high-anxiety[93]. G carriers of rs2270007 (C:79%, G:21%) of the *CRHR2* gene showed a worse overall response to citalopram[147]. Furthermore, a SNP (rs10473984; G:87%, T:13%) within the *CRHBP* locus, encoding the CRH-binding protein which inactivates corticotrophin releasing factor (CRF), showed a significant association with both remission and reduction in depressive symptoms in response to citalopram. The T-allele of this SNP was associated with poorer treatment outcome in 2 out of the 3 ethnic subsamples (African-American and Hispanic), despite large differences in minor allele frequency. This association was more pronounced in patients with features of anxious depression[148].

Box 5: Hypothalamic system

Upon stress exposure, the hypothalamus secretes corticotrophin releasing factor (CRF), arginine vasopressin (Avp) and oxytocin (Oxt), triggering the release of proopiomelanocortin (POMC) from the pituitary, that is cleaved in, among others, adrenocorticotropic-hormone (ACTH). ACTH, on its turn, stimulates the secretion of the stress hormones cortisol (humans) and corticosterone (rodents). Feedback control of the HPA-axis is mediated by glucocorticoid (GR) and mineralocorticoid (MR) receptors in selective brain areas. CRF is not only found in the hypothalamus, but also in cortical and limbic structures, in particular high levels are found in the central nucleus of the amygdala[335] where it plays a role in stress response[336] and addiction[337]. Besides the HPA axis, the hypothalamus is involved in many more processes. The hypothalamus regulates homeostasis; it has regulatory areas for thirst, hunger, body temperature, water balance, and blood pressure, and links the nervous system to the endocrine system. Furthermore, the hypothalamus is involved in the reproductive system by secretion of gonadotropin releasing hormone (GnRH). GnRH stimulates the release of follicle-stimulating hormone by the pituitary gland, which, in turn, stimulates the release of estrogens (by the follicles) or testosterone (by the testes).

Animals-in vivo

More data on SSRI effects of the expression of HPA-axis components have been obtained in experimental animals. Starting at the level of the paraventricular nucleus (PVN) in the hypothalamus, male mice acutely treated with fluoxetine (10 mg/kg) exhibited no changes in arginine vasopressin (*Avp*), oxytocin (*Oxt*) and *Crf* mRNA expression, but female wild-type animals showed increased PVN *Avp* and *Oxt* mRNA levels[95]. Upon chronic fluoxetine treatment, amygdala *Crf* gene expression was decreased[45], and Brady and colleagues[64] observed that chronic fluoxetine (5 mg/kg, i.p.) treatment for 2-8 weeks decreased *Crf* mRNA

levels by 30-48% in the PVN of the hypothalamus. The decreases occurred at 8 weeks but not at 2 weeks. Proopiomelanocortin (*Pomc*) mRNA levels in the anterior pituitary and plasma ACTH levels were not altered after 2 or 8 weeks of drug treatment. However, Jensen and colleagues[150] showed that a daily treatment with citalopram (10 mg/kg, s.c.) for 14 days decreased the expression of *Pomc* mRNA in the anterior pituitary by 40%. Furthermore, it has been demonstrated that 5 mg/kg fluoxetine (i.p.) increased the level of mineralocorticoid receptor (MR) mRNA in the hippocampus after 8 weeks of drug administration[64]. In addition, mice treated with fluoxetine (5 mg/kg, i.p.) for 5 weeks showed decreased glucocorticoid receptor (GR) gene (*Nr3c1*) expression in the prefrontal cortex, amygdala, locus coeruleus and DRN[45]. Effects of fluoxetine on the expression of GRs may be age-dependent, as chronic (1 month) fluoxetine (10 mg/kg, p.o.) treatment increased *Nr3c1* mRNA in the hippocampus of 4 months old rats, and MR (*Nr3c2*) mRNA levels in CA2 of 24 month old rats[96]. Together these data suggest that chronic SSRI exposure decreases the activity of the HPA-axis through enhancing GR/MR feedback regulation, although the decreased *Nr3c1* mRNA levels found by Heydendael *et al.*[45] may also reflect diminished corticosterone secretion due to decreased CRF and POMC levels and therefore a reduced need for feedback regulation.

The HPA-axis is implicated not only in stress responses, but also in feeding. In female goldfish, chronic fluoxetine treatment (5 µg/gr, 13 days) reduced body weight and food intake, and induced an up-regulation of both *crf1* and neuropeptide Y (*npy*) mRNAs in the hypothalamus. In the telencephalon there was a decrease of *npy* mRNA and an increase in amphetamine-regulated transcript (CART)-1 mRNA. No changes in tachykinin mRNA were observed in either the hypothalamus or telencephalon[104]. NPY exerts orexogenic effects and is under inhibitory regulation of CRF[338]. Their up-regulation may therefore reflect a compensatory mechanism to counteract the decrease in food intake. Chronic fluoxetine (10 mg/kg, i.p., 2 weeks) administration in male obese Zucker rats decreased food intake along with increased *Pomc* expression and reduced melanocortin receptor 4 (*Mc4r*) expression in the hypothalamus, without changes in Agouti gene related peptide (*Agrp*) mRNA levels. These findings indicate that the melanocortin system is involved in the anorectic mechanism of fluoxetine[100].

Again another hypothalamic system is the reproductive system driven by gonadal hormones. A female goldfish study showed that fluoxetine (5 µg/gr, 14 days) significantly reduced the expression of estrogen receptor (ER)beta1 mRNA in both the hypothalamus and the telencephalon, and ERalpha mRNA in the telencephalon. Fluoxetine had no effect on the expression of ERbeta2 mRNA in the hypothalamus or telencephalon. Microarray analysis identified isotocin, a neuropeptide that stimulates reproductive behavior in fish, as a candidate gene affected by fluoxetine treatment. Real-time RT-PCR verified that isotocin mRNA was down-regulated in the hypothalamus and the telencephalon. Intraperitoneal injection of isotocin (1 µg/g) increased plasma estradiol, providing a potential link between changes in isotocin gene expression and decreased circulating estrogen in fluoxetine-injected fish[105]. An association between estradiol and SSRI treatment was also observed in a study

in which rats were ovariectomized and supplemented with estradiol (E(2)) and thereafter withdrawn from the E(2) supplementation in half of the animals. It was found that in acute sertraline-treated (10 mg/kg, i.p.) rats, E(2) decreased *Htr2a* receptor mRNA, and E(2)-withdrawal increased *Htr1a*, *Htr2a* and *Htr2c* receptor mRNA. These effects were not seen after imipramine (tricyclic antidepressant; TCA) treatment[339], implying that E(2) synergizes behaviorally and neurochemically with acute SSRI administration.

Knockout animals

When the acute effects to 10 mg/kg fluoxetine (s.c.) on HPA-axis activation and the associated release of vasopressin (which together with CRF stimulates the secretion of ACTH from the anterior pituitary) were measured in *Avpr1b* knockout mice and wild-type controls, significantly attenuated plasma ACTH and CORT levels were noted in the knockout mice compared to their control counterparts. *Avp, Oxt* and *Crf* mRNA expression in the PVN did not change in acute fluoxetine (10 mg/kg, s.c.)-treated male *Avpr1b* knockout or wild-type mice. In contrast, fluoxetine treatment increased PVN *Avp* mRNA levels in female *Avpr1b* wild-type but not knockout animals. PVN *Oxt* mRNA levels increased in fluoxetine-treated female mice of both genotypes. The data suggests that the *Avpr1b* gene is required to sex-dependently drive the HPA axis response to acute antidepressant treatment[95]. In glucocorticoid receptor-impaired mice hippocampal cell proliferation and BDNF mRNA expression were down-regulated, and these alterations were reversed by chronic fluoxetine (10 mg/kg, i.p., 21 days) treatment. Fluoxetine did not reverse GR (*Nr3c1*) mRNA down-regulation, nor did it increase the survival of newly formed cells in the ventral part of the hippocampus[340], which implies that the increase in *Bdnf* mRNA in fluoxetine exposed mice may be mediated by the activation of several pathways which are independent from GR, however this does not exclude that a link between GR and BDNF exists.

Inflammatory system

Inflammation is an early response of the immune system to injury often caused by infection. An inflammatory response is produced by eicosanoids and cytokines, which are released by injured or infected cells and recruit immune cells to the site of infection and promote healing of any damaged tissue. Cytokines include interleukins (communication between white blood cells), chemokines (promoting chemotaxis) and interferons (anti-viral effects). Common eicosanoids include prostaglandins (fever and dilation of blood vessels) and leukotrienes (attracting leukocytes). Proinflammatory cytokines may provoke changes in brain structure and function, leading to the development of depression. Cytokines can directly modulate pathways implicated in the aetiology of depression, like neurotransmission, the HPA axis and hippocampal neurogenesis. Proinflammatory cytokines can down-regulate 5-HT synthesis by lowering the availability of tryptophan (the substrate for serotonin synthesis) through activation of indoleamine-2,3-dioxygenase (IDO)[341-343], and increasing neurotoxic kynurenine

metabolites such as 3-hydroxy kynurenine and quinolinic acid[344]. In addition, it is shown that cytokines can enhance the reuptake of 5-HT[345]. Proinflammatory cytokines also reduces the synthesis[346] and enhance the reuptake of dopamine[347]. The effect of cytokines on the HPA-axis has been attributed to glucocorticoid receptor (GR) resistance. Inflammatory cytokines and their signaling pathways including mitogen-activated protein kinases, nuclear factor-kappaB, signal transducers and activators of transcription, and cyclooxygenase have been found to inhibit GR function resulting in overactivity of the HPA-axis[348]. Furthermore, it has been shown that neuroinflammation inhibits neurogenesis[349,350] and that inflammatory blockade with a non-steroidal anti-inflammatory drug restores neurogenesis[349] .

Human-polymorphisms

Regarding inflammation, studies mainly address polymorphisms in interleukins. It is shown that MDD patients who were homozygous for the-511T allele (rs16944; T:53%, C:47%) of the interleukin-1 beta (IL-1β) gene had a trend of less severity of depressive symptoms and more favorable fluoxetine therapeutic response than -511C carriers[351]. Furthermore, Tadić and colleagues[352] investigated the possible association of the IL-1β C-511T promoter polymorphism and the interleukin-1 receptor antagonist (IL-1Ra) (86bp)n VNTR polymorphism with antidepressant response to paroxetine treatment. They show that patients homozygous for the IL-1β-511T-allele had a significantly faster and more pronounced response to paroxetine treatment than IL-1β-511C-allele carriers, while the IL-1Ra VNTR was not associated with paroxetine treatment response. Another genome-wide pharmacogenetics study showed an association between a polymorphism in the interleukin-11 gene (rs1126757) and response to escitalopram[200]. Finally, gene polymorphisms affecting SSRI efficacy were found in *IDO*, which is a gene associated with both the 5-HT pathway and the immune system. Two SNPs (rs2929115 and rs2929116), located between 26 kb and 28 kb downstream of the *IDO2* gene, showed an association with citalopram response[132].

Human-lymphoblasts

In line with the inflammation hypothesis of depression, implying that inflammatory responses in the brain leads to depression-related symptoms[344], it was found in leukocytes of depressed patients that mRNA expression of the cytokines IL-1beta, IL-6, (interferon) IFNgamma, (tumor necrosis factor) TNFalpha, and 5-HTT were higher compared to those of the healthy controls. The higher level of mRNA expressions of IFNgamma and 5-HTT diminished after fluoxetine treatment. Furthermore, there was a positive correlation between 5-HTT and cytokine mRNA expression in all participants, which suggested that pro-inflammatory cytokines and 5-HTT might play critical roles in the pathogenesis of major depression and that their levels were affected by chronic treatment with 5-HTT inhibitors[106]. The cytokine-5-HT interaction in depression may be mediated by IDO, as mentioned before. Furthermore, one study employed a genome-wide profiling approach (microarrays) in human T-lymphocytes treated with paroxetine and sertraline and revealed down-regulation of *CDC2* (Cell division cycle 2,

G1 to S and G2 to M), *CDC6* (cell division cycle 6 homolog (Saccharomyces cerevisiae), *TNFSF8* (Tumor necrosis factor (ligand) superfamily,member 8), *IL2RA* (Interleukin 2 receptor, alpha Transcription factor Dp-1), *LMAN1* (Lectin, mannosebinding, 1), *STAT1* (Signal transducer and activator of transcription 1), which are involved in inflammation, as well as in gene proliferation, cancer and apoptosis[165]. Together, there is evidence that genes involved in the immune system are affected by SSRI treatment. However, it has to be taken into consideration that these studies are performed in lymphocytes and that gene expression and regulatory mechanisms can be highly tissue specific.

Miscellaneous genetic effects of SSRIs

Human polymorphisms

Some miscellaneous effects of SSRIs have also been reported. It was found that polymorphisms in the *CLOCK* gene (rs3736544, rs1801260, rs3749474; GTT, GCC and TTC, respectively) were significantly associated with fluvoxamine's therapeutic response in Japanese depressed patients[214]. This observation supports the idea that MDD is associated with disruptions in biological circadian rhythms, and that SSRIs have circadian properties. Furthermore, polymorphisms in pharmacokinetic genes (*CYP2D6, ABCB1, CYP2C19, CYP3A4, CYP450, and CYP3A5*) failed to affect the efficacy of SSRIs[353-355].

Animals-in vivo

Among the miscellaneous effects of SSRIs found in animal studies is the observation that chronic citalopram (30 mg/kg/day, 4 weeks, via drinking water) treatment prevented stress-induced up-regulation of synaptic vesicle glycoprotein 2b (*Sv2b*) and glial N-myc downstream-regulated gene 2 mRNA levels, involved in exocytosis of synaptic vesicles and neurite outgrowth, respectively. Furthermore, increased mRNA levels for neuron-specific enolase (NSE, *Eno2* gene) were found in both stressed and unstressed citalopram treated animals in the dorsal raphe nucleus[134]. NSE has neurotrophic and neuroprotective effects on different types of cultured neurons[356,357]. The neuromodulator somatostatin, which inhibits the release of hormones from the pituitary gland and regulates dopamine release in the striatum[358], was also found to be modulated by SSRI treatment. A single citalopram injection (10 mg/kg, i.p.) significantly reduced somatostatin levels in the striatum and nucleus accumbens after 4 but not 1, 8, or 24 h. No changes were found in hippocampus. In addition, it was found that the K+-evoked overflow of somatostatin-like immunoreactivity from striatal slices was significantly increased 1 h after a single injection of citalopram and was still higher, although not significantly, 4 h after the drug injection. Levels of preprosomatostatin mRNA were unchanged in striatum and accumbens 1 and 4 h after a single drug administration. When rats were treated with citalopram (10 mg/kg i.p.) twice daily for 14 days, the levels of somatostatin and its mRNA were significantly decreased in the striatum but not in other brain

regions 24 h after the last dose. No change was found in the basal or K+-evoked overflow of somatostatin-like immunoreactivity at 1, 4, and 24 h after the last drug injection[156].

Age-dependent effects

Whereas the safety of SSRIs has been well established in adults, safety is not guaranteed in children and adolescents. Treatment during adolescence may increase the risk for suicide[359] and prenatal treatment results in adverse outcomes, such as premature birth[360], neonatal cardiovascular abnormalities[361], and pulmonary hypertension[362]. Furthermore, neonatal and adolescent SSRI exposure leads to paradoxical anxiety- and depression-like features in later life of rats and mice[20,363].

Animals-in vivo

To unravel the potential molecular mechanism underlying the age-dependent effects of SSRIs postnatal day 13, 21, 28 and adult rats were treated with escitalopram (2-15 mg/kg, i.p.) for four days, twice a day and effects on BDNF and its receptor TrkB were studied. *Bdnf* mRNA and BDNF protein levels, as well as TrkB (*Ntrk2*) mRNA levels, were significantly increased in postnatal day 13 pups after escitalopram treatment as compared to control. However, desipramine, a TCA, failed to increase either *Bdnf* or *Ntrk2* expression. The failure of desipramine to increase BDNF and TrkB levels in juvenile rats is consistent with the lack of efficacy of desipramine in children and adolescents and may relate to the fact that the serotonergic nervous system matures earlier than the noradrenergic system[199]. Another study focused on the neuronal PAS domain protein 4 (*Npas4*), one of the transcription factors regulating BDNF. The *Npas4* gene expression was significantly decreased in animals that were exposed to chronic fluoxetine during gestation, but fluoxetine treatment of adult rats did not result in a significant change in the mRNA levels of the transcription factor[88].

Animals-in vitro

Cultured cells are also highly informative to dissect the age (development)-dependent effects of SSRIs, as they allow the visualization of cell proliferation and differentiation. Using a mesencephalic neural cell line, mes-c-myc A1 (A1), producing 5-HT and expressing 5-HTT and both peripheral TPH1 and CNS-specific TPH2, it was reported that fluoxetine (10 μM) modulates the expression of serotonergic markers depending on the differentiation status of the cells. Interestingly, long-term (7 days), but not short-term (1 day), fluoxetine treatment selectively modulated mRNA levels of *Tph2*, only in differentiated A1 cells. Furthermore, SSRIs selectively decreased the proliferation rate of undifferentiated A1 cells, whereas they had no effects on NIH-3T3 fibroblasts proliferation, showing that the differentiation stage of cells influences SSRI responsiveness[364]. In embryonic stem (ES) cells, fluoxetine (0-1 μM) was found to decrease the viability of the cultured cells as well as their ability to differentiate into cardiomyocytes, possibly by inducing fluctuations in endodermal marker gene expression

(*Gata6, Ttr, Afp,* and *Alb*) during ES cell differentiation[107]. Fluoxetine (0-1 μM) treatment was also found to enhance the expression of glial marker genes (*Gfap, Olig2, Dm20,* and *S100b*) following neural differentiation, to increase the population of glial cells. The expression of neuronal markers during ES cell differentiation into neural cells (*Tuj1, Syp, Th,* and *Nurr1*), on the other hand, was inhibited by fluoxetine treatment, suggesting that fluoxetine facilitates the differentiation of mouse ES cells into glial cell lineage, which may affect fetal neural development[65].

In the human cerebellum, the proliferation of cerebellar granule cells continues until the 11th postnatal month and could be influenced in infants by breastfeeding-delivered SSRIs. To address this issue rat postnatal cerebellar neural progenitors were exposed to fluoxetine (0.001–20 mM) for 72 hours. RT-PCR and immunostaining revealed the expression of 5-HTT, 5-HT$_{1A}$ receptors, TPH, and 5-HT, indicating that these cells are capable of producing 5-HT and controlling 5-HT release. Protracted *in vitro* fluoxetine treatment increased cell proliferation and differentiation. The proliferative effect of fluoxetine was abolished by the selective 5-HT$_{1A}$ receptor antagonist WAY-100635. Furthermore, fluoxetine induced activation of both CREB protein and extracellular signal-regulated protein kinases (ERK1/2), which was abolished by the selective inhibitor of MAP kinase kinase (MEK) 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene (U0126), and increased cyclin D1 expression. All these effects were prevented by WAY-100635, indicating that rat postnatal cerebellum contains neural progenitors capable of proliferating and differentiating in response to fluoxetine exposure, possibly through the activation of 5-HT$_{1A}$ receptors[109].

In summary, SSRI treatment has differential effects on mRNA expression across age groups and cell stages. The effects on cell proliferation and differentiation may affect fetal neural development and plasticity. The age dependent behavioral effects as described above might be caused by these underlying molecular differences. However, the precise mechanisms causing the differences in molecular and behavioral effects across developmental stages are not clear and elucidating these mechanisms would be a great challenge.

Regulation of gene expression

Studies discussed so far show that SSRI efficacy is modified by various polymorphisms in human genes, and that SSRIs alter the mRNA expression of multiple genes belonging to various pathways. What is not yet clear so far is whether the transcription of genes is under tight epigenetic and microRNA (miRNA) control. Epigenetic modifications represent modifications of DNA and histones that regulate various genomic functions occurring without changes in DNA sequence. DNA methylation involves the transfer of a methyl group to cytosine residues at cytosine-phosphate-guanosine units, which is catalyzed by the enzyme DNA methyltransferase. DNA methylation at specific gene promoters is an important mechanism to control gene transcription. Histone modifications which are mediated by histone acetyltransferases/deacetylases and histone methyltransferases/demethylases

determine the accessibility of the chromatin for the transcription machinery. Studies on the epigenetic mechanisms underlying the antidepressant potential effect of SSRIs are marginal, in contrast to those of TCA's and selective noradrenaline reuptake inhibitors. One study reported that P11 (*S100a10*) mRNA levels were decreased in the prefrontal cortex of the Flinders Sensitive Line (genetic rodent model of depression), along with higher DNA methylation at its promoter. This hypermethylation was reversed to wild-type levels after chronic escitalopram treatment. Escitalopram caused hypomethylation of *S100a10* and increased *S100a10* gene expression, as well as a reduction in DNA methyltransferases (*Dnmt1* and *Dnmt3a*) involved in the maintenance of forebrain DNA methylation[202]. P11 is involved in the intracellular trafficking of transmembrane proteins to the cell surface[365], including the 5-HT_{1B} and 5-HT_4 receptor, and thereby plays an important role in 5-HT signaling[366]. Studies investigating the epigenetic mechanisms underlying the antidepressant effects of TCA treatment are more abundant. Genome wide analysis in mice demonstrated that chronic defeat stress causes widespread and long-lasting changes in gene regulation, including alterations in repressive histone methylation (dimethylK9/K27-H3), which is reversed by imipramine treatment[367]. In another study, Tsankova and colleagues[368] showed that in the hippocampus *Bdnf* gene expression is down-regulated, coincided with histone methylation of H3K27 after chronic social defeat stress. Chronic imipramine treatment reversed this down-regulation with increasing acetylation of H3 at these promoters. Furthermore, they show that this hyperacetylation by chronic imipramine was associated with a selective downregulation of histone deacetylase 5 (*Hdac5*). One study showed evidence for SSRI-induced histone acetylation at the *Bdnf* promoter, although not in relation to depression[369]. Since TCAs and SSRIs both act on the serotonergic system[370] and this system strongly interacts with BDNF, it is likely that SSRI treatment causes similar epigenetic changes at the *Bdnf* promoter. In line with the increase in H3 acetylation by antidepressant treatment, administration of HDAC inhibitors also resulted in antidepressant-like effects[371]. Furthermore, Sales et al.[372] showed that (systemic and intra-hippocampal) administration of DNA methyltransferase inhibitors also results in antidepressant-like effects.

miRNAs are short ribonucleic acid (RNA) molecules binding to complementary sequences on target messenger RNA transcripts (mRNAs), usually resulting in translational repression or target degradation and gene silencing. Their role in mediating SSRI effects was shown in an elegant study revealing that chronic fluoxetine treatment in mice led to increased miR-16 levels in serotonergic raphe neurons and reduced *Slc6a4* expression. Furthermore, raphe exposed to fluoxetine were found to release the neurotrophic factor S100β, which turned on the expression of serotonergic functions in noradrenergic neurons of the locus coeruleus[108]. In addition, changes in the expression of determinants of mRNA translation may mediate SSRI effects. As such, chronic fluoxetine treatment (10 mg/kg, i.p.) induced hyperphosphorylation of eukaryotic elongation factor 2 (eEF2 - Thr56) in prefrontal cortex, hippocampus and dentate gyrus of rats and hyperphosphorylation of eukaryotic initiation factor 4E (eIF4E - Ser209) specifically in the dentate gyrus. Acute fluoxetine treatment had

no effect on translational factor activity[373]. In summary, SSRIs not only directly target 5-HT receptor linked signal transduction pathways and gene expression, they may also regulate gene expression by modifying miRNAs and epigenetic processes.

Lessons from other antidepressants

We proposed several pathways which contribute to the antidepressant effects of SSRIs. In this section we will briefly discuss the effect of non-SSRI antidepressant drugs on these pathways. If a pathway is affected by several antidepressant drugs, it is more likely that this pathway plays a major role in inducing antidepressant effects. Tricyclic antidepressants (TCAs) and monoamine oxidase inhibitors (MAOIs) both influence the serotonergic system and also other neurotransmitter systems: MAOIs by blocking the enzyme monoamine oxidase which breaks down the neurotransmitters dopamine, serotonin, and norepinephrine, and TCAs by blocking the reuptake of norepinephrine and serotonin. This emphasizes the importance of the serotonergic system, and also the norepinephrine and dopamine systems, in inducing antidepressant effects. In addition, these non-SSRI antidepressants also affect some of the other pathways discussed in the previous sections. TCAs, like imipramine, can activate glial cell line-derived neurotrophic factor[374] and increase the number of hippocampal synapses and neurons[375], indicating neuroplastic effects. Furthermore, it is shown that TCA treatment reduces HPA-axis responsivity[376] and inflammation[377]. In addition, TCAs induce epigenetic modifications. As mentioned before, imipramine can reverse stress-induced histone methylation (dimethylK9/K27-H3) and increase acetylation of H3. MAOIs, like tranylcypromine, are linked to neuroplasticity by increasing hippocampal BDNF expression[320]. Also, HPA-axis responsivity[378] and the production of proinflammatory cytokines[379,380] are reduced by MAOI treatment. Since SSRIs and these non-SSRI drugs are all directly affecting the serotonergic system, it is possible that the effects on other systems are “side effects” caused by downstream pathways of the serotonergic system and do not contribute to the antidepressant effect. Drugs or compounds with antidepressant effects not directly acting on the serotonergic system can give more insights into the antidepressant potential of these pathways. Regarding inflammation, there are a few studies showing that anti-inflammatory drugs can reduced depressive-like symptoms. For example, it is shown that cyclooxygenase inhibition - an enzyme that is responsible for formation of prostaglandins - is beneficial in treating depression[381,382], however there are also studies showing negative results[383]. Furthermore, selective CRH1 antagonists, acting on HPA-axis signaling, show antidepressant effects[384,385] and are also known for normalization of REM sleep[386]. Also, infusion of BDNF into the dentate gyrus of hippocampus in rats produced an antidepressant effect, showing evidence for the involvement of plasticity in antidepressant effects[387]. Finally, as mentioned before, epigenetic modifiers, like HDAC inhibitors and DNA methyltransferase inhibitors, exert antidepressant effects.

Conclusion

Our review of current literature on the genetic targets of SSRIs provides several crucial insights:

1] 5-HTT gene (*SLC6A4*) variation in humans consistently influences the antidepressant efficacy of SSRIs, which makes sense for 5-HTT being the main target of SSRIs. More precisely, the low activity (short; S) variant of the 5-HTTLPR associated with reduced *SLC6A4* gene transcription[388] is linked to a poor responsiveness to SSRIs in depressed patients across ethnicities. In this context, it is striking that in animal studies *Slc6a4* mRNA levels are generally not affected by chronic SSRI treatment. Rather, these studies reveal that posttranslational changes in 5-HTT modify SSRI responsiveness, for instance at the level of internalization. Taken together, this indicates that especially the amount of 5-HTT in the cell surface is linked to SSRI responsiveness.

2] Gene variations in many of the 5-HT receptor genes (*HTR2C*, *HTR1A*, *HTR1B*) are associated with antidepressant efficacy. In addition, *in vivo* animal studies showed down-regulation of *Htr2c* and presynaptic *Htr1a* and *Htr1b* mRNA levels by chronic SSRI treatment. Furthermore, the actions of SSRIs on sleep (5-HT_7 and 5-HT_{1a}), nausea (5-HT_{3b}), feeding (5-$HT_{2c,}$ 5-HT_{1B}) and gastrointestinal side effects (5-$HT_{2a)}$ are essentially mediated by specific 5-HT receptors, indicating that 5-HT receptors and their different downstream pathways play a major role in the antidepressant and side effects of SSRIs.

3] Besides the serotonergic system, SSRIs can interact with other (neurotransmitter) systems, like the DA system, the HPA system and the immune system. DA neurotransmission is up-regulated by SSRIs, which is in line with the fact that SSRIs have a weak affinity for the DA transporter[370]. The activity of the HPA axis, involved in controlling stress responses, is down-regulated by SSRIs, which indicates that besides the serotonergic system the HPA-axis is an important contributor to the antidepressant effects of SSRIs. Furthermore, SSRIs influence the immune system by down-regulation of cytokines. It has been shown that proinflammatory cytokines play an important role in the development of depression by enhancing the reuptake of 5-HT[345] and DA[347] and reducing the synthesis of 5-HT (by influencing IDO[342]) and DA (by influencing BH4, a cofactor for tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis[389]). In addition to the modulation of neurotransmitter function, proinflammatory cytokines also activate the HPA axis by increasing the release of CRF[348,390]. In summary, down-regulation of the proinflammatory cytokines plays an important role in mediating the antidepressant effects of SSRI treatment by inhibition of the HPA axis, enhancing 5-HT and DA synthesis and inhibition of 5-HT and DA reuptake. The importance of proinflammatory cytokines in depression raises the question whether drugs that have a central anti-inflammatory action might foster a new generation of antidepressants.

4] SSRIs influence neuroplasticity by affecting the expression level of several neuroplasticity factors. In particular the effect of SSRIs on BDNF has been extensively studied. Polymorphisms in the *BDNF* gene and in other genes in the BDNF pathway are associated with SSRI treatment outcome and expression studies showed an increase in BDNF expression upon SSRI treatment,

which is time dependent and region specific. Since depression is shown to be associated with decreased plasticity[391,392], SSRI-induced enhanced neuronal plasticity should contribute to the recovery from depression.

5] Little is known so far about the effects of SSRIs on epigenetic modifications. One study showed that SSRIs can reverse DNA hypermethylation, namely of the P11 gene (*S100a10*). In contrast, studies investigating the epigenetic effects of TCA treatment are more abundant and show that chromatin remodeling is an important mechanism in stress responses and antidepressant treatment. However, the exact mechanism by which stress and antidepressants, in particular SSRIs, converge on chromatin still needs to be elucidated.

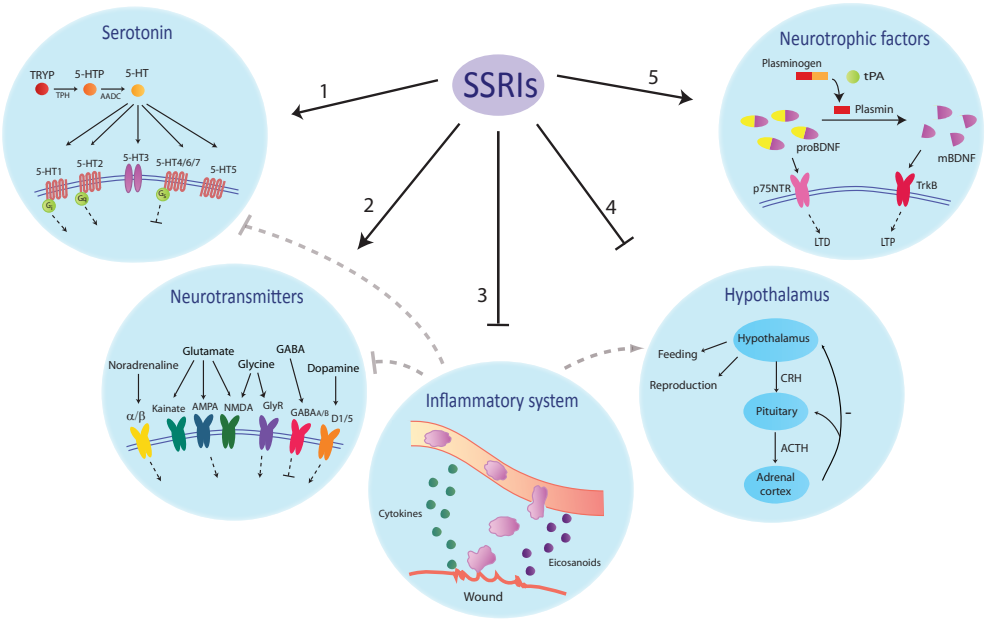

Figure 2. Pathways affected by SSRI treatment. Overview of the pathways affected by SSRI treatment. (1) The main target of SSRIs is the serotonin transporter. Blocking of this transporter will cause an increase in extracellular serotonin levels and subsequently an increase in activation of serotonin receptors and their downstream pathways. (2) SSRIs can also activate other neurotransmitter systems by low-affinity binding to other transporters. (3) SSRIs influence the inflammatory system by down-regulation of cytokines. (4) The activity of the HPA axis, involved in controlling stress responses, is down-regulated by SSRIs and also other hypothalamic processes, like regulation of hunger and reproduction, are influenced by SSRI treatment. (5) SSRIs influence neuroplasticity by affecting the expression level of several neuroplasticity factors. A well-known factor that is influenced by SSRI treatment is the brain derived neurotrophic factor (BDNF). In addition, the inflammatory system, the serotonin system, the dopamine system and the HPA axis are interacting with each other resulting in indirect effects (dashed lines). The down-regulation of the proinflammatory cytokines plays a role in mediating the antidepressant effects of SSRI treatment by inhibition of the HPA axis and influencing 5-HT and DA synthesis and reuptake.

6] SSRI treatment has differential effects on mRNA expression across age groups and cell stages. SSRI-induced age-dependent effects are also shown at behavioral level and might be a consequence of these underlying molecular differences. The precise mechanisms causing the differences in molecular and behavioral effects across developmental stages are not clear. Possibly, age differences in 5-HT receptor expression and their downstream signaling pathways, as well as in epigenetic regulation, might play a role in these age-dependent effects.

In conclusion, SSRIs exert their antidepressive effects via a wide variety of pathways in addition to the serotonergic pathway (Figure 2). However, to obtain an overview of the exact molecular mechanisms contributing to the antidepressive effects, some unanswered questions still need to be elucidated. Especially studies investigating the molecular mechanisms underlying the age-dependent effects and studies addressing the epigenetic effects of SSRIs might give more insights in the working mechanism(s) of SSRIs.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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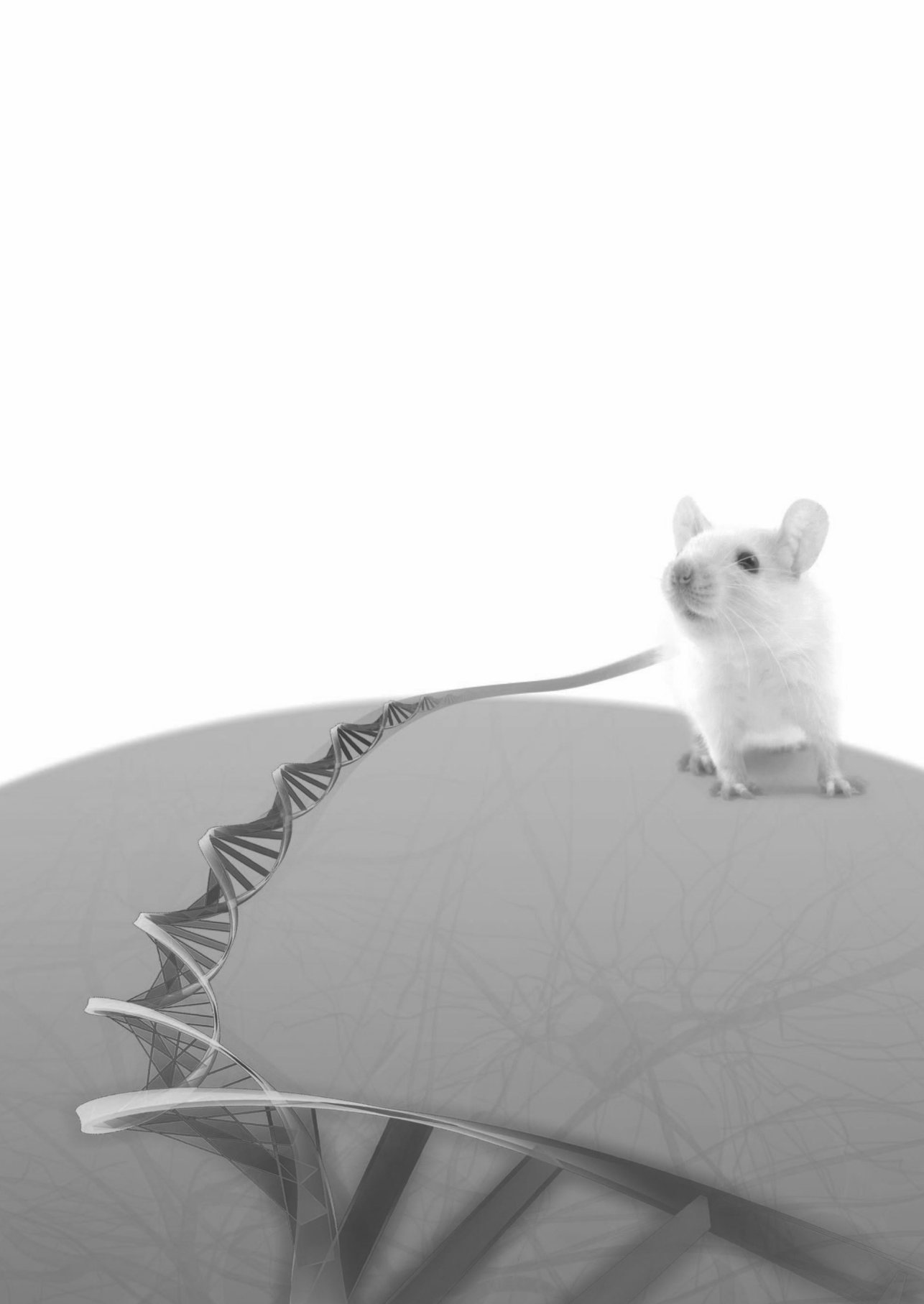

3

Long-term consequences of chronic fluoxetine exposure on the expression of myelination-related genes in the rat hippocampus

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Abstract

The selective serotonin reuptake inhibitor (SSRI) fluoxetine is widely prescribed for the treatment of symptoms related to a variety of psychiatric disorders. After chronic SSRI treatment, some symptoms remediate on the long-term, but the underlying mechanisms are not yet well understood. Here we studied the long-term consequences (40 days after treatment) of chronic fluoxetine exposure on genome-wide gene expression. During the treatment period, we measured body weight; and one week after treatment, cessation behavior in an SSRI-sensitive anxiety test was assessed. Gene expression was assessed in hippocampal tissue of adult rats using transcriptome analysis and several differentially expressed genes were validated in independent samples. Gene ontology analysis showed that upregulated genes induced by chronic fluoxetine exposure were significantly enriched for genes involved in myelination. We also investigated the expression of myelination-related genes in adult rats exposed to fluoxetine at early-life and found two myelination-related genes (*Transferrin (Tf)* and *Ciliary neurotrophic factor (Cntf)*) that were downregulated by chronic fluoxetine exposure. *Cntf*, a neurotrophic factor involved in myelination, showed regulation in opposite direction in the adult versus neonatally fluoxetine-exposed groups. Expression of myelination-related genes correlated negatively with anxiety-like behavior in both adult and neonatally fluoxetine-exposed rats. In conclusion, our data reveal that chronic fluoxetine exposure causes on the long-term changes in expression of genes involved in myelination, a process that shapes brain connectivity and contributes to symptoms of psychiatric disorders.

Introduction

Selective serotonin reuptake inhibitors (SSRIs) are widely prescribed for the treatment of a variety of psychiatric disorders, such as depression[1,2], bipolar affective disorder[3], anxiety-related disorders (obsessive compulsive disorder (OCD), post-traumatic stress disorder (PTSD)) [4,5], aggression[6] and autism spectrum disorder (ASD)[7,8]. All these disorders have symptoms related to, among others, negative affectivity, which might be the symptom responsive to SSRI treatment. Clinical studies have shown that there are many side effects upon chronic administration of SSRIs, like sexual dysfunction[9], suppression of REM sleep[10], nausea[11,12], decreased appetite[13] and deterioration of symptoms (for example, aggression)[14,15], indicating that optimization of chronic treatment of affective disorders is needed. In addition, some patients remain free of symptoms after discontinuation of SSRI treatment, whereas in others the symptoms reoccur[16,17]. Studies in generalized social anxiety disorder patients showed that within 24 weeks after discontinuation of SSRI treatment 40-50% of the patients (receiving placebo after stopping SSRI) relapsed[18,19]. For major depressive disorder, the cumulative probability of suffering a recurrence of major depression within 2 years after discontinuation of SSRIs was 60% for people aged 60 years or older[20]. Hence, the effects of SSRIs are not always sufficient to warrant long-term remission. To further improve the efficacy of SSRIs, there is an urgent need to unravel the mechanisms contributing to the long-term effects of chronic SSRI exposure.

The primary target of SSRIs is the serotonin transporter (5-HTT); its blockade by SSRIs leads to increases in extracellular serotonin (5-HT) levels. According to classic literature, chronic rises in 5-HT levels would contribute to the therapeutic effects of SSRIs[21], acting through 5-HT receptors. For example, antagonism of 5-HT_{2C} and 5-HT_{7} results in significantly increased therapeutic effects of SSRIs[22-25] and SSRI/5-HT_{1A} antagonist co-administration leads to increased extracellular 5-HT levels and enhanced antidepressant responses[26,27]. However, these and other 5-HT receptors are also implicated in the side effects of SSRIs, like sexual dysfunction (5-HT_{1A})[28], sleep disturbances (5-HT_{7}[29] and 5-HT_{1A}[30]), nausea (5-HT_{3B}[31]) and decreased appetite (5-HT_{2C}[32], 5-HT_{1B}[33]). Although 5-HT receptors have a key role in the short-term effects of chronic SSRI exposure, it is likely that pathways downstream of the receptors are important for the long-term effects of chronic SSRI exposure.

Recently, several gene expression studies in rodents have shown that SSRI exposure can affect a wide variety of pathways in addition to the serotonergic system. It has been shown that SSRI treatment during adulthood alters gene expression levels of hypothalamic hormones, neurotrophic factors, inflammatory factors and components of non-serotonergic neurotransmitter systems[34-36]. Especially the inflammatory factors might play a central role in mediating the effects of SSRIs, because downregulation of proinflammatory cytokines can inhibit HPA axis function (facilitating stress reduction), enhance 5-HT and dopamine synthesis and inhibit 5-HT and dopamine reuptake[34]. Yet, these findings reflect short-term effects (24 hours after the last treatment) of chronic SSRI exposure. The long-term consequences of SSRI exposure on gene expression are so far not well understood and might provide additional

information about the long-term adaptations that contribute to the remediation of disease symptoms after stopping medication.

Studies addressing the long-term consequences of perinatal SSRI exposure may provide hints regarding potential mechanisms by which SSRIs exert their long-term effects. In humans and rodents, there is evidence that perinatal SSRI exposure increases the likelihood of symptoms related to autism[37-41] in the offspring. This seemingly contrasts the use of SSRIs in the treatment of autism during adulthood. In addition, in rodents perinatally exposed to SSRIs there is evidence for 'paradoxical' anxiety- and depression-like symptoms at adulthood[42-44]. Because adult and perinatal SSRI exposure is associated with comparable effects on the serotonergic system, like increases in 5-HT levels, reductions in 5-HT transporter expression[45,46] and desensitization of 5-HT_{1A} receptors[47,48], the 'paradoxical' outcomes of perinatal SSRI exposure cannot be explained by 5-HT levels (alone). Critically, during development, 5-HT not only acts as neurotransmitter, but also as a neurotrophic factor. Specifically, during early brain development, 5-HT steers neurodevelopmental processes like neuronal outgrowth and migration processes[49-51]. Studies have shown that 5-HT affects embryonic interneuron migration[51] and also affects organization of axonal projections of excitatory spiny stellate and pyramidal cells in the barrel cortex[52]. These data show that 5-HT affects the outgrowth and migration of non-serotonergic neurons. As the brain is highly plastic during early development, rises in 5-HT levels induced by perinatal SSRI exposure can have outcomes that are substantially different from adult SSRI exposure. Nonetheless, studies focusing on early-life SSRI exposure could lead to potential targets of the long-term chronic SSRI exposure during adulthood. For example, SSRI exposure during brain development can disturb myelin sheath formation at adulthood[40] and there is also evidence that SSRI treatment at adulthood can cause changes in white matter microstructure, which consists mainly of myelinated axons[53]. Furthermore, both adult and developmental SSRI exposure can affect hippocampal neurogenesis at adulthood[54,55].

To more concretely elucidate the long-term effects of chronic SSRI exposure during adulthood, we investigated the long-term consequences of chronic fluoxetine (12 mg/kg) versus vehicle treatment during adulthood (postnatal day (PND) 67-88) on gene expression in the hippocampus, a brain region that is highly responsive to SSRIs[55,56] and implicated in psychiatric disorders characterized by affective changes like anxiety[57,58], bipolar affective disorder[59], aggression[60] and depression[61]. It has for example been shown that the hippocampus is directly involved in the mediation of unconditioned anxiety-related responses in animals[57]. We measured body weight during treatment, as fluoxetine is known to exert anorectic effects[62,63]. In addition, we measured anxiety-like behavior in the novelty-suppressed feeding test (NSFT), which is highly sensitive to SSRIs[42,43,64-66]. We studied genome-wide gene expression using transcriptome analysis (RNA-seq) in hippocampal tissue of fluoxetine and vehicle-exposed rats 40 days after the last treatment. Differentially regulated genes were validated by quantitative reverse transcription PCR (RT-qPCR) analysis using independent samples. Gene ontology analysis showed that the majority of upregulated genes had a function in

myelination. To assess whether genes involved in myelination were also affected by early-life exposure to fluoxetine, we performed qPCR analysis on genes involved in myelination in a group of rats neonatally exposed to fluoxetine or vehicle. Finally, we performed correlational analysis between anxiety-like behavior and messenger RNA (mRNA) expression.

Materials and methods

Animals

Male Wistar rats (*Rattus norvegicus*) were obtained from Charles River (Cologne, Germany) and used for experiments after at least seven days of acclimatization. All animals were housed per two in standard Macrolon® type 3 cages in temperature-controlled rooms (21°C±1°C) under a standard 12/12-h day/night cycle (lights on at 07:00 a.m.) with food (Sniff, long cut pellet, Bio Services, Uden, The Netherlands) and water available *ad libitum*. Environmental conditions (for example, housing, light conditions (80 lux), noise level) were carefully controlled as these conditions can strongly influence stress levels in rats[67,68]. Three groups of animals were used in this study. In each group, the rats were randomly assigned to a treatment. The investigator was not blinded to the group allocations when performing the experiments, because effects of fluoxetine on the body weight and behavior were clearly visible. Group 1 was treated at adulthood with fluoxetine or vehicle (n = 12 per treatment), used for body weight measurements during treatment, tested in the NSFT and decapitated to collect hippocampal tissue for RT-qPCR validation; group 2 was treated at adulthood with fluoxetine or vehicle (n = 4 per treatment) and used for RNA-seq experiments. Finally, group 3 consisted of adult female Sprague Dawley rats neonatally exposed to fluoxetine or vehicle (PND 1 to 21) via osmotic minipumps implanted in the mothers. Their hippocampal tissue was obtained from Maastricht University (fluoxetine n = 6, vehicle n = 7) and used for RT-qPCR analysis. Figure 1 provides a schematic representation of the experimental timeline for each group. For behavior experiments, 12 animals per group were used, because this is the minimum required to achieve sufficient statistical power to establish significant differences ($\alpha = 0.05$ and $\beta = 0.20$). For genome-wide gene expression analysis we used two biological replicates. All experiments were carried out according to the guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council 2003), the principles of laboratory animal care, as well as the Dutch law concerning animal welfare.

Drug treatment

Rats from group 1 and 2 received fluoxetine (12 mg/kg/day, as used by Olivier and colleagues[42]) or vehicle by oral gavage from PND 67 to 88 in a volume of 5 ml/kg. Fluoxetine was purchased from the Pharmacy of the Radboud University Nijmegen Medical Centre, The Netherlands and dissolved in distilled water. As a vehicle, 1% methylcellulose (Genfarma B.V. Maarssen, The Netherlands) was used, which was the constituent of the fluoxetine pills. Body weight was monitored daily throughout the treatment. Rats from group 3 received fluoxetine via the milk of the dams. Minipumps were implanted subcutaneously in the dorsal region of the

dams on PND1 and filled with either fluoxetine–HCl (Fagron, Belgium) dissolved in vehicle (50% propylenediol in saline; 5 mg/kg/day), or with vehicle, as previously described[69].

Novelty-suppressed feeding test (NSFT)

The NSFT was performed as described before[42]. In short, after food deprivation, male rats (PND 95) of group 1 were placed in one corner of an open arena (50×50 cm) containing clean wood chip bedding with in the center a filter paper containing a food pellet. The latency (s) to start an eating episode was recorded (maximum time was 900 s). After each rat the arena was cleaned with ethanol (70%) and dried thoroughly to prevent transmission of olfactory cues.

Transcriptome sequencing

Rats within group 2 were sacrificed at PND 128, brains were removed and stored at-80°C. The hippocampus was dissected by punching from 300 micron frozen brain slices, and tissue from two rats was pooled for total RNA isolation and cDNA synthesis. DNA samples were prepared for RNA-seq by end repair, adapter ligation, size selection and amplification. After quality control of DNA libraries, samples were sequenced (36 bp, single read) with the Illumina Genome Analyzer IIx platform. Sequences were aligned to the rat rn4 reference genome[70] and further analyzed using Genomatix software (www.genomatix.de). The Database for Annotation, Visualization and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov/) was used for gene ontology (GO) analysis. RNA-seq validation was performed by RT-qPCR analysis in an independent group of rats (group 1). See supplementary information for detailed information about the transcriptome analysis and primer sequences (Supplementary Table 1).

Quantitative Reverse Transcription PCR

Hippocampal tissue of rats within group 3 was crunched in liquid nitrogen. RNA was isolated (RNeasy lipid tissue kit; QIAGEN, Venlo, The Netherlands) and cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad, Veenendaal, The Netherlands) according to manufacturer's protocols. The qPCR reactions were performed in 7500 Fast Real Time PCR System (Applied Biosystems, Foster City, CA, U.S.A) using the SYBR Green fluorescence quantification system (GoTaq® qPCR Master Mix, Promega, Leiden, The Netherlands). See supplementary information for detailed information about the RT-qPCR method.

Statistical analysis

Statistical analysis of the data was carried out using the IBM Corp. Statistical Package for the Social Sciences (SPSS) version 20.0 (IBM Corp, Armonk, NY, USA). The Shapiro-Wilk test was used to check for normal distributions. Independent samples *t* tests were used for normally distributed data (corrected p-value was used when equal variance was not assumed) and Mann-Whitney U tests for non-normal distributions. Body weight was analyzed by repeated measures analysis of variance (ANOVA) and further analyzed per day using independent samples *t* tests. Spearman correlations were performed for the correlational analysis between

behavior tests and mRNA expression. Outliers (data points further than 3 interquartile ranges from the nearer edge of the box plot) were excluded from the analysis. Independent samples *t* tests and correlations were performed two-sided. No adjustments for multiple comparison was applied for the RNA-seq. We performed qPCR validations afterwards to validate the RNA-seq results. The level of statistical significance was set at $p<0.05$ in all tests.

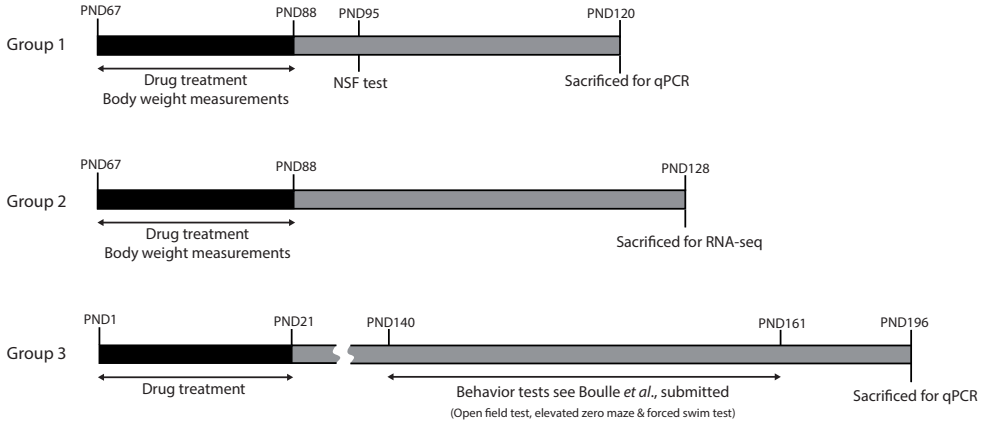

Figure 1. Schematic representation of experimental timeline. Male Wistar rats, group 1 (n=12 per treatment) and 2 (n=4 per treatment), were treated with fluoxetine or vehicle from postnatal day (PND) 67 to 88. During the treatment period body weight was measured every day. In group 1, anxiety-like behavior was tested on PND 95 using the novelty-suppressed feeding test (NSFT). Groups 1 and 2 were sacrificed by decapitation on PND 120 and PND 128, respectively and used for mRNA expression analysis using hippocampal tissue. Group 3 (fluoxetine n=6, vehicle n=7) was used to investigate the effect of chronic fluoxetine exposure on hippocampal mRNA expression in Sprague Dawley rats. For neonatal exposure dams were treated during the postpartum period from PND 1 to 21. At PND 21, pups were weaned and group-housed for further examination (two rats per cage). Anxiety- and depression-related behavior was analyzed from PND 140 onwards (in the order as written in the figure) and rats were sacrificed by decapitation at PND 196. mRNA, messenger RNA; qPCR, quantitative PCR.

Results

Body weight and anxiety-like behavior in response to adult fluoxetine exposure

Body weight was measured daily during the treatment period. All rats received a daily oral administration of fluoxetine or vehicle from PND 67 to 88 (Figure 1). As shown in Figure 2A, starting weight in group 1 was not different between fluoxetine and vehicle groups ($t_{(1,22)}$=0.26; p=0.796). Repeated measures ANOVA revealed that fluoxetine significantly reduced adult body weight gain ($F_{(1,22)}$=43.37; $p<0.01$). Independent samples *t* tests indicated that the reduction in body weight gain was significant ($p<0.05$) from day 4 of the treatment and further on. Vehicle-exposed rats grew on average from 295.5 g on the first day of treatment to 350.3 g on the last day of treatment, while fluoxetine-exposed rats grew on average from 294.8 g on the first day of treatment to 320.2 g on the last day of treatment (see Supplementary Table 2 for all body weight values). Similar results were obtained for group 2 (data not shown). Anxiety-like behavior was measured one week after treatment using the

NSFT. We found that adult fluoxetine-exposed rats exhibited a shorter latency to start eating compared to vehicle-exposed animals ($t_{(1,19)}$=2.32; p<0.05; Figure 2B, Supplementary Table 2). Both decreased weight gain during chronic fluoxetine exposure[71] and a shorter latency to start eating in the NSFT after chronic fluoxetine exposure[64-66,72] are consistent with previous findings in stressed and unstressed rats.

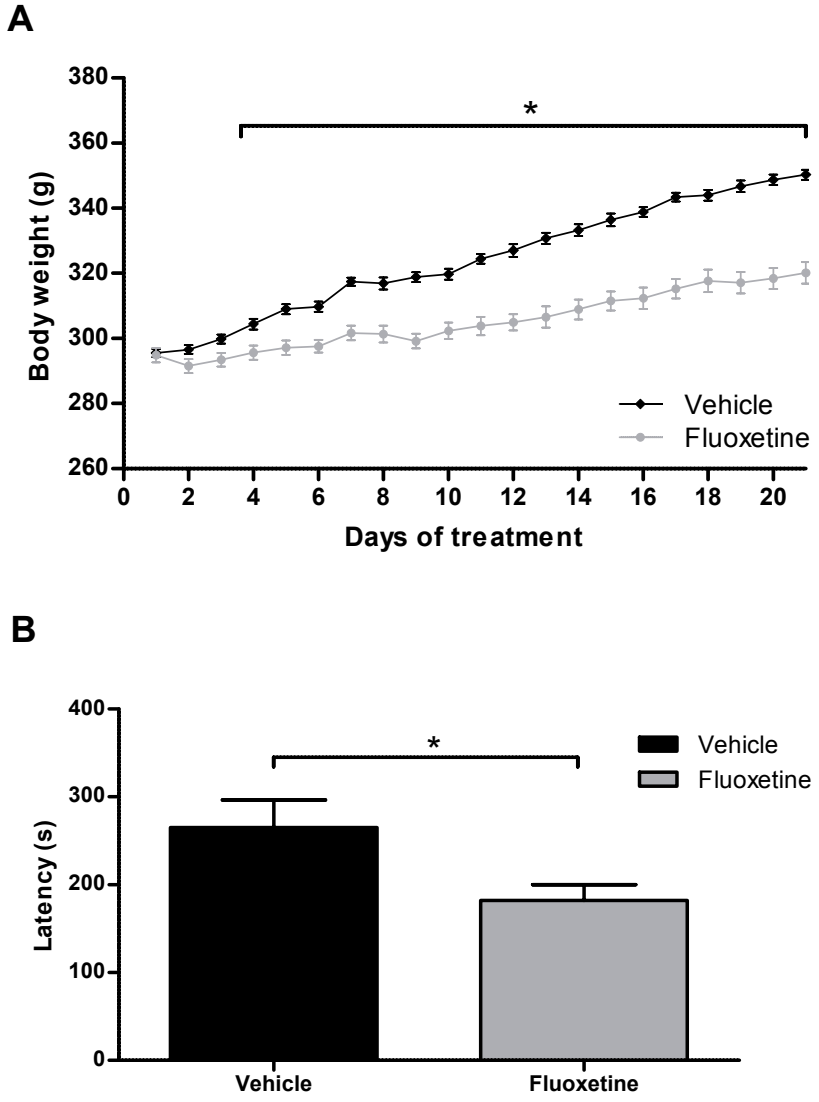

Figure 2. Fluoxetine in adult exposed Wistar rats reduces body weight and latency to start eating in a novel environment. A: Body weight in fluoxetine and vehicle treated (postnatal day (PND) 67 to 88) adult male rats (n=12/group) measured during the treatment period. Data are presented as mean ± S.E.M. of body weight (g). B: Latency to start eating in a novel environment tested in fluoxetine and vehicle treated adult male rats on PND 95. Data are presented as mean + S.E.M. of latency (s) to start eating. *p<0.05

Long-term consequences of adult chronic fluoxetine exposure on genome-wide mRNA expression patterns in the hippocampus

To investigate which genetic pathways have a role in the long-term effects of chronic SSRI exposure, RNA-seq analysis was performed using hippocampal tissue of fluoxetine- and vehicle-exposed rats (two rats pooled per sample, two samples per treatment group). Genes with a fold change >1.5 fold and a p-value <0.05 were considered as differentially regulated genes. Analysis of the samples resulted in 258 genes that were significantly upregulated and 218 genes that were significantly downregulated by fluoxetine treatment (Figure 3A, Online Supplementary Table 3). Some genes show overlap with a study in mice chronically treated with fluoxetine (see green marked genes in Online Supplementary Table 3)[73].

To functionally categorize the differentially expressed genes, GO analysis was performed. The most significantly enriched GO-terms in the list of upregulated genes induced by adult fluoxetine treatment are all involved in glia cell development and myelination (Table 1). Examples of upregulated genes involved in glia cell development are *zinc finger protein 488 (Znf488), proteolipid protein 1 (Plp1), ciliary neurotrophic factor (Cntf), NK6 homeobox 2 (Nkx6-2)* and *POU class 3 homeobox 1 (Pou3f1)*. For the genes downregulated after adult fluoxetine treatment the most significantly enriched GO-term was 'response to abiotic (non-living) stimulus'. An underlying and more specific GO-term that was also significantly enriched is 'response to temperature stimulus (an abiotic stimulus)', including genes such as *adrenoceptor beta 2* (*Adrb2), nitric oxide synthase 1 (Nos1), caspase 8 (Casp8), transient receptor potential cation channel, subfamily V, member 3 (Trpv3), interleukin 1 beta (Il1b), chemokine (C-X-C motif) ligand 12 (Cxcl12)* and *protein kinase C, delta (Prkcd)*. See Online Supplementary table 4 for a complete list of significantly enriched GO-terms including the genes linked to these terms.

Validation of the adult RNA-seq data was performed by RT-qPCR analysis in independent biological replicates (n=11-12 per treatment). For validation, we selected 12 differentially regulated genes (five up- and seven downregulated) on the basis of p-value (p<0.05), fold change (>1.5) and expression profile using the WIG files. Five genes, *olfactomedin 1* (*Olfm1, U*=31.00, p<0.05; downregulated)*, adenylate cyclase 1 (Adcy1, U*=33.00, p<0.05; downregulated)*, neurotensin (Nts,U*=25.00, p<0.05; upregulated)*, Cntf* (*U*=26.00; p<0.05; upregulated) and *claudin 11 (Cldn11, U*=25.00, p<0.05; upregulated), showed a significant change in mRNA expression in the same direction as in the RNA-seq data (Figure 3B). Interestingly, three out of the five significantly upregulated genes in RNA-seq were significantly upregulated in RT-qPCR analysis and the other two genes also showed a change in the right direction, that is, upregulation in the fluoxetine-exposed rats. However, the majority of the genes downregulated in the RNA-seq where not changed in the RT-qPCR analyses, indicating that the upregulated genes were more consistent among independent experiments. Of the upregulated genes, *Cntf, Cldn11* and *Tspan2 (p=0.17)* are involved in myelination[74-76], indicating that myelination is one mechanism involved in the long-term effects of SSRI exposure.

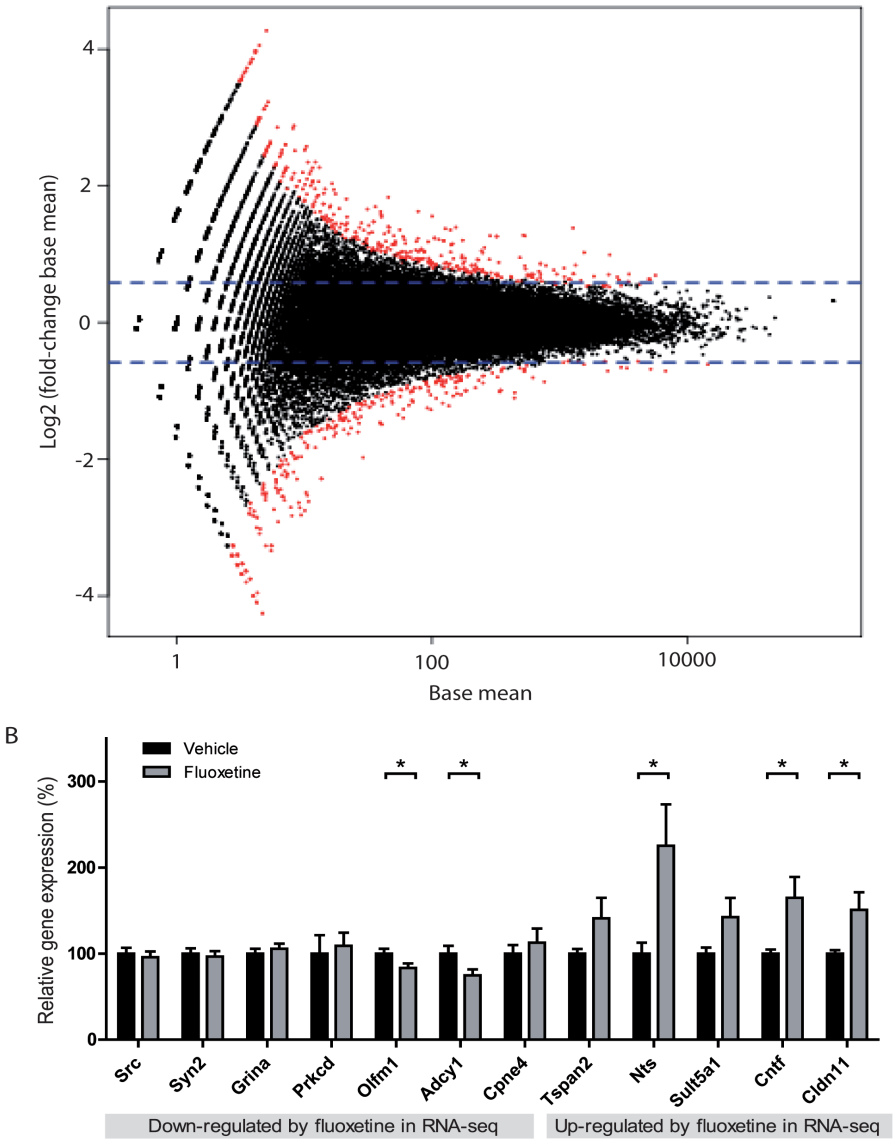

Figure 3. Gene expression in adult fluoxetine-exposed male Wistar rats. A: RNA-seq analysis was performed using hippocampal tissue of fluoxetine- and vehicle-exposed rats, two rats pooled per sample, two samples per treatment group. Fold change scatter plot showing fold change in expression (base mean) in fluoxetine-treated versus vehicle-treated rats (y-axis) against expression level (x-axis). Differentially regulated genes are genes with fold change threshold > 1.5 (log2 fold change > 0.58, blue dashed line) and p-value < 0.05 (colored in red). Red dots above the upper blue dashed line are upregulated genes (258 genes) and red dots below the lower blue dashed line are downregulated genes (218 genes). B: Validation of RNA-seq results by quantitative RT-PCR (RT-qPCR) analysis in independent biological replicates. Quantitative RT-PCR was performed on hippocampal RNA of adult fluoxetine and vehicle treated (postnatal (PND) day 67 to 88) rats (n=12 per treatment). Based on RNA-seq data, 7 genes downregulated (left side in figure) and 5 genes upregulated (right side in figure) by fluoxetine exposure were selected for RT-qPCR validation. Data are normalized for *Ywhaz* and *Hprt* mRNA levels and are presented as mean + S.E.M. of relative gene expression (% of vehicle group). Genes differentially expressed in RT-qPCR are marked with * (=p<0.05).

Long-term consequences of neonatal chronic fluoxetine exposure on hippocampal mRNA expression

As GO analysis showed that upregulated genes are enriched for genes involved in myelination, we investigated whether myelin-linked genes were also affected in adult rats neonatally exposed to fluoxetine. We had access to hippocampal tissue of adult rats exposed to fluoxetine or vehicle from PND 1 to 21 and performed RT-qPCR analysis for several genes involved in myelination (based on Aston and colleagues[77]). Expression of *Cntf,* a gene also detected and validated in the RNA-seq experiment, was significantly reduced in response to neonatal fluoxetine exposure compared to vehicle (U=6.00, p<0.05). In addition, a significant reduction after fluoxetine exposure was found for *transferrin* (*Tf, U*=4.00, p<0.05; Figure 4). Consistent with the long-term effects on gene expression after chronic fluoxetine treatment during adulthood, these data show that genes associated with myelination are also involved in the long-term effects of neonatal SSRI exposure, but in the opposite direction.

Correlation between behavior and expression of myelination-related genes

To investigate whether the anxiolytic effect of chronic SSRI exposure (see section 'Body weight and anxiety-like behavior in response to adult fluoxetine exposure') is related to the altered expression of myelination-related genes, we performed a correlational analysis. Group 1 was used for both the NSFT and qPCR validations, which enables correlational analysis between latency to start eating and mRNA expression (Supplementary Figure 1). Interestingly, we found a negative correlation ($r_{(18)}$=-0.529, p<0.05) between *Cldn11* mRNA expression and the latency to start eating in the NSFT. In addition, we found a trend for a negative correlation between *Tspan2* mRNA expression and latency to start eating ($r_{(18)}$=-0.412, p<0.1).

We also performed a correlational analysis using data (anxiety-like behavior in an open field test (OFT, results see Boulle and colleagues[78]) and expression analysis of myelination-related genes) derived from the neonatally fluoxetine-exposed rats (group 3). In the OFT, time spent in the corner (OFCo) and time spent in the center (OFCe) of the open field were measured, in which OFCo is a measure for anxiety-like behavior and OFCe a measure for anxiolytic-like behavior. We found that OFCo correlated negatively with mRNA expression of *Cldn11* ($r_{(11)}$=-0.736, p<0.05), *Cnp* ($r_{(11)}$=-0.682, p<0.05), *Plp1* (ex3-5) ($r_{(11)}$=-0.827, p<0.05) and *Plp1* (ex2-3) ($r_{(11)}$=-0.800, p<0.05). In addition, *Mag* mRNA expression showed a trend for a negative correlation with OFCo ($r_{(11)}$=-0.555, p<0.1). Finally, a trend for a positive correlation with OFCe was found for mRNA expression of *Cldn11* ($r_{(11)}$=0.582, p<0.1), *Plp1* (ex3-5) ($r_{(11)}$=0.527, p<0.1) and *Plp1* (ex2-3) ($r_{(11)}$=0.536, p<0.1) and *Mog* ($r_{(11)}$=0.573, p<0.1). See Supplementary Figure 1-3 for a complete overview of the correlation data.

Taken together, these data indicate that a higher expression of myelination-related genes is linked to anxiolytic-like behavior in both the NSFT in adult fluoxetine-exposed rats and the OFT in the neonatally fluoxetine-exposed rats.

Table 1. Significantly enriched GO-terms (biological process) affected by adult fluoxetine treatment

Factor	DAVID ID	GO-term	# genes	P-value	Fold enrichment
Upregulated by fluoxetine	GO:0021782	Glial cell development	5	4.4E-4	13.7
	GO:0010001	Glial cell differentiation	6	7.1E-4	8.4
	GO:0042063	Gliogenesis	6	2.0E-3	6.7
	GO:0042391	Regulation of membrane potential	8	2.5E-3	4.3
	GO:0008366	Axon ensheathment	5	2.6E-3	8.6
	GO:0007272	Ensheathment of neurons	5	2.6E-3	8.6
	GO:0001508	Regulation of action potential	6	3.1E-3	6.0
	GO:0033205	Cytokinesis during cell cycle	3	3.9E-3	30.8
Downregulated by fluoxetine	GO:0009628	Response to abiotic stimulus	15	8.2E-5	3.5
	GO:0051931	Regulation of sensory perception	4	7.1E-4	22.1
	GO:0051930	Regulation of sensory perception of pain	4	7.1E-4	22.1
	GO:0009266	Response to temperature stimulus	7	7.7E-4	6.4
	GO:0035239	Tube morphogenesis	8	1.3E-3	4.8
	GO:0043044	ATP-dependent chromatin remodeling	3	2.3E-3	40.2
	GO:0044236	Multicellular organismal metabolic process	4	2.8E-3	13.9
	GO:0060562	Epithelial tube morphogenesis	6	4.0E-3	5.7

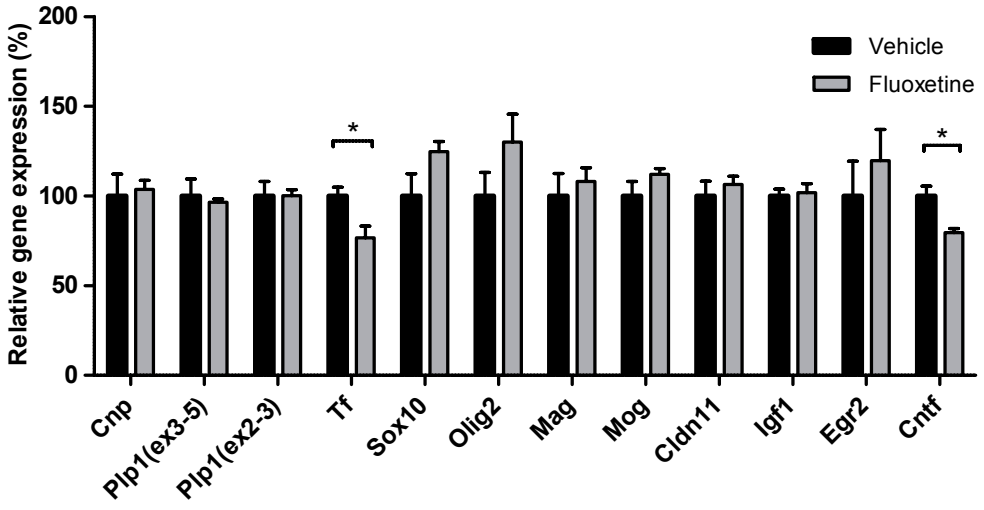

Figure 4. Hippocampal mRNA expression levels in neonatally fluoxetine-exposed female Sprague Dawley rats. Quantitative RT-PCR analysis was performed on hippocampal RNA of adult rats (fluoxetine n=6, vehicle n=7) neonatally exposed (postnatal day (PND) 1 to 21) to fluoxetine or vehicle. Data are normalized for *Ywhaz* and *Hprt* mRNA levels and are presented as mean + S.E.M. of relative gene expression (% of vehicle group). *p<0.05

Discussion

In this study, we demonstrate, using a genome-wide approach, that 40 days after chronic fluoxetine treatment in adult rats mRNA levels of myelination-related genes were significantly upregulated in the hippocampus. Interestingly, in an independent group of rats we observed that chronic neonatal fluoxetine exposure downregulated myelination-related genes. We specifically observed that the myelination-related *Cntf* gene was upregulated in adult fluoxetine-exposed rats and downregulated in neonatally fluoxetine-exposed rats. In addition, we observed a negative correlation between expression of myelination-related genes and anxiety-like behavior in both the adult and neonatally fluoxetine-exposed rats. These data suggest that chronic SSRI exposure exerts its long-term effects, amongst others, by affecting myelination processes.

There are other studies in rodents showing genome-wide gene expression differences after adult fluoxetine treatment, but so far they all focused on short-term effects by investigating gene expression one day after the last fluoxetine administration[73,79-81]. The present finding that myelination-related genes were affected more than 40 days after chronic SSRI exposure, both in early-life and adulthood, is important given that it elucidates the neurobiological mechanisms contributing to the development of (early-life exposure) and recovery from (adult exposure) psychiatric disorders. Interestingly, there is overlap in differentially regulated genes between studies focusing on short-term effects and our study about long-term effects. For instance, Samuels and colleagues[73] performed a microarray study using dentate gyrus tissue from 24 hours after treatment cessation of adult mice chronically treated with fluoxetine and identified 8 upregulated and 20 downregulated genes that overlap with our findings (see green marked genes in Online Supplementary Table 3). Genes affected in both short- and long-term studies might play a crucial role in inducing and maintaining the antidepressant state. It is not likely that effects of fluoxetine withdrawal are seen in our expression data, because these effects occur short after withdrawal and will not last for 40 days.

RNA-seq validation by qPCR showed that the upregulated genes were more consistent among independent experiments. We were unable to validate 5 out of the 7 downregulated genes, therefore we focused on the upregulated genes. The GO analysis of genes upregulated by chronic fluoxetine exposure in adulthood revealed that the majority of these genes have a function in myelination. In addition, we found a correlation between the latency to start eating in the SSRI-sensitive NSFT and gene expression of myelination-related genes (*Cldn11,* and a trend for *Tspan2*), which strengthens our findings. Interestingly, a wide range of psychiatric disorders responsive to SSRI treatment, including depression, bipolar affective disorder, autism, OCD, PTSD and ASD have been associated with defects in white matter, which consists mainly of myelinated axons[82,83]. A first link between mood disorders and myelin was shown by Aston and colleagues[77]. They studied gene expression in the temporal cortex of major depressive disorder patients and found a decreased expression of genes encoding structural components of myelin (for example, *2',3'-cyclic nucleotide 3' phosphodiesterase*

(CNP), myelin-associated glycoprotein (MAG), myelin oligodendrocyte glycoproteinn (MOG), PLP1) and genes involved in myelin formation (e.g. *TF, SRY (sex determining region Y)-box 10 (SOX10)*). We showed in our RNA-seq experiment that the SSRI fluoxetine increases the expression of genes linked to myelination in the hippocampus. Interestingly, we did not find the same genes as Aston and colleagues found in the temporal cortex (gene expression might be brain region dependent), but we did find genes (*Cntf, Cldn11*) influencing the same process. Genes interacting with each other (*SOX10* and *Cntf*[84]) and genes with similar functions regarding myelination (*PLP1* and *Cldn11*[85]) are found in the study by Aston and colleagues and our RNA-seq experiment. Moreover, in OCD patients, abnormalities of myelin integrity have been found that were partially reversed by SSRI treatment[53]. Taken together, these findings suggest that myelination is dysregulated in several psychiatric disorders and can be regulated by antidepressants, like fluoxetine.

In hippocampal tissue of neonatally fluoxetine-exposed rats, we found that *Cntf* was downregulated, which directly opposed the finding that adult fluoxetine exposure upregulated this very same gene. Thus, the same gene, *Cntf*, was affected in opposite direction by chronic fluoxetine exposure in early-life and adulthood. This is consistent with the growing amount of experimental evidence that early-life SSRI exposure leads to 'paradoxical' autism-, anxiety- and depression-like symptoms in later life[1,42-44]. In agreement, our neonatally fluoxetine-exposed rats showed increased depression-like behavior (forced swim test) at adulthood compared with the neonatally vehicle-exposed rats[78]. Furthermore, Boulle and colleagues showed that neonatal fluoxetine exposure decreased *Bdnf* IV expression in hippocampus[78], while others observed increased *Bdnf* expression in hippocampus of adult fluoxetine-exposed rats 24 hours after treatment cessation[86]. *Cldn11* expression, which was upregulated in the adult fluoxetine-exposed group, was not found to be regulated in the opposite direction (downregulated) in the neonatally fluoxetine-exposed group.

The decrease in expression of the two myelin-linked genes after early-life fluoxetine exposure is in line with the findings of Simpson and colleagues[40]. They showed that early-life SSRI exposure (citalopram) disturbs myelin sheath formation and decreases interhemispheric connectivity by 50%. In addition, high levels of serotonin can lead to aberrant oligodendrocyte development and myelination deficits *in vitro*[87]. Our results of the RT-qPCR in hippocampus tissue of early-life fluoxetine-exposed rats suggest that gene expression of myelination-related genes was also affected by SSRIs. Notably, our adult and neonatally fluoxetine-exposed groups differed in fluoxetine dose, strain and gender, making it possible that the opposite finding was driven by these factors rather than neonatal versus adult fluoxetine exposure. However, our finding that expression of the myelination-related *Cldn11* gene and anxiety correlated negatively in both the adult and neonatally fluoxetine exposed rats does not support this. Given that changes in myelination have been reported by others after both neonatal[40] and adult[53] SSRI exposure, it is more likely that our findings are the result of fluoxetine exposure at different ages.

The *Cntf* gene, coding for ciliary neurotrophic factor, is the only gene differentially

regulated in all our experimental groups. CNTF is a neurotrophic factor produced by astrocytes, which supports the proliferation[88] and survival[89-91] of oligodendrocyte precursors and regulates myelination[74]. Studies have shown that CNTF can mediate stroke-induced adult CNS neurogenesis[92] and that CNTF injection can increase remyelination in cuprizone-induced multiple sclerosis mice[93], supporting the role of CNTF as a neurotrophic factor and as a myelin regulator. In the hippocampus *Cntf* is strongest expressed in the dentate gyrus and CA1 regions[94]. The dentate gyrus is important for adult neurogenesis and therefore *Cntf* expression in this region fits well with its role in neurogenesis. Studies have shown that CNTF is essential for the formation and/or maintenance of the neurogenic subgranular zone in the adult dentate gyrus[95]. How fluoxetine targets myelination-related genes is still unclear. Based on literature, we propose a potential pathway, but this is highly speculative (see Supplementary Figure 4). In short, fluoxetine stimulates the 5-HT_{2B} receptor on astrocytes resulting in activation of its downstream signaling cascades[96], which potentially can lead to release of CNTF. The released CNTF can activate astrocytes and these astrocytes then release an astrocyte specific factor (>30 kDa), which promotes proliferation and survival of oligodendrocyte precursor cells[97] and maturation of oligodendrocytes[90,98]. Of further interest, *Cntf-/-* mice display increased anxiety- and depression-like behavior[99]. These findings are in line with the reduced *Cntf* expression we found in the group of rats exposed to fluoxetine at early-life, which also showed increased depression-like behavior[78]. *Cldn11* expression is upregulated by adult chronic fluoxetine exposure and showed a negative correlation with anxiety-like behavior in the NSFT. In the neonatally fluoxetine-exposed rats, *Cldn11* expression also showed a negative correlation with anxiety-like behavior (OFCo) in the open field test, despite the absence of significant differences between the treatment groups in the OFT and the expression analysis. *Cldn11* codes for Claudin-11, which is a major component of myelin and forms tight junctions within myelin sheaths[100]. Downregulation of *Cldn11* has been found in bipolar affective disorder patients[101]. Also *Plp1* and *Cnp* showed a negative correlation with anxiety-like behavior (OFCo) in the open field test. Taken together, the correlations indicate that a higher expression of myelination-related genes results in anxiolytic-like behavior.

In this study, we found that fluoxetine can cause long-term changes in the expression of myelination-related genes. However, a potential limitation of the present study is that we used a homogenate of hippocampus cells and there are different cell types in the hippocampus tissue. Selecting a specific cell type using fluorescence-activated cell sorting might give more insights in the gene expression per cell type, although it is notable that mRNA levels correlated with behavior. Another limitation of this study is that the fluoxetine dose differs between the prenatally (5 mg/kg/day) and adult (12 mg/kg/day) exposed groups. However, studies have shown that exposure to higher doses of fluoxetine early in life (10-20 mg/kg/day) affects anxiety-like behavior (e.g. open field test) in the same way a seen for 5 mg/kg[102,103]. Furthermore, studies using a lower dose of fluoxetine in adulthood (5 mg/kg/day) showed a similar effect on anxiety-like behavior in the NSFT as shown in this study for 12 mg/kg/day[65]. In the future it is relevant to explore whether fluoxetine exposure will give

similar results in models for psychiatric disorders responsive to SSRIs. Given that the effect of SSRIs in the NSFT is the same in healthy[64,65] and stressed[66,72] animals it is likely that also gene expression patterns will be similar. Finally, we measured gene expression, and it remains to be established whether our findings translate to changes in protein levels and myelination. As a next step in biology, evidence of changes in myelination will further support our findings. As such, it has already been demonstrated that SSRI treatment can have consequences for myelination[40,53,104].

In conclusion, we show that adult and neonatal chronic fluoxetine exposure cause on the long-term changes in hippocampal expression of ciliary neurotrophic factor and other genes linked to myelination, a process that shapes brain connectivity and could contribute to the remediation of symptoms of psychiatric disorders, like anxiety.

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Conflict of interest

The authors declare no conflict of interest.

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Supplementary information

Supplementary materials and methods

RNA extraction and Double-Stranded cDNA Synthesis

Animals of group 2 were sacrificed at PND 128, brains were removed and immediately placed on dry ice and stored at -80°C. Hippocampal tissue was dissected in 8 consecutive slices of 200 µm using a 2 mm punch needle. Tissue from 2 rats was pooled for total RNA isolation with QIAzol (RNeasy lipid tissue kit; QIAGEN, Venlo, The Netherlands) according to the manufacturer's recommendations. Total RNA was subjected to two rounds of poly(A) selection (Oligotex mRNA Mini Kit; QIAGEN), followed by DNaseI treatment (QIAGEN) and fragmentation by hydrolysis (5× fragmentation buffer: 200mM Tris acetate, pH8.2, 500mM potassium acetate and 150mM magnesium acetate) at 94°C for 90 s. Fragmented mRNA was purified (RNeasy MinElute Kit; QIAGEN) and used for cDNA synthesis with 5 µg random hexamers by Superscript III Reverse Transcriptase (Invitrogen Life technologies, Bleiswijk, The Netherlands). Double stranded cDNA synthesis was performed in second strand buffer (Invitrogen) according to the manufacturer's recommendations and purified using the Minelute Reaction Cleanup Kit (QIAGEN) according to the manufacturer's protocol.

Sequencing

DNA samples were prepared for RNA-seq by end repair of 20 ng DNA as measured by Qubit dsDNA HS (Invitrogen). Adaptors were ligated to DNA fragments, followed by size selection (~300 bp) and 14 cycles of PCR amplification. Quality control of DNA libraries prepared for sequencing was made by qPCR and by running the products on a Bioanalyzer (Bio-Rad, Veenendaal, The Netherlands). Cluster generation and sequencing (36 bp, single read) was performed with the Illumina Genome Analyzer IIx (GAIIx) platform according to standard Illumina protocols. Samples were sequenced to a depth of approximately 15 million uniquely mapped tags per sample. Sequences were aligned to the rat rn4 reference genome with the Illumina Analysis Pipeline allowing one mismatch. Only the tags aligning to one position on the genome were considered for further analysis. The output data were converted to Browser Extensible Data (BED) files for downstream analysis and Wiggle (WIG) files for viewing.

Data analysis

RNA-seq data were analyzed using Genomatix software (www.genomatix.de). The number of sequence reads for each transcript was quantified and additionally a standardized normalized expression (NE) value per transcript was calculated (based on the number of reads located in the exons of the transcript and normalized to the length of the transcript and the density of the data set). NE values of vehicle and fluoxetine samples (group 2) were used to calculate fold change values for each transcript. Genes were identified as differentially expressed if they showed a DESeq[1] p-value < 0.05 and fold change (FC) > 1.5 (among two biological replicates for both fluoxetine and vehicle treatment). The Database for Annotation, Visualization and

Integrated Discovery (DAVID, http://david.abcc .ncifcrf.gov/) was used for gene ontology (GO) analysis.

Quantitative Reverse Transcription PCR

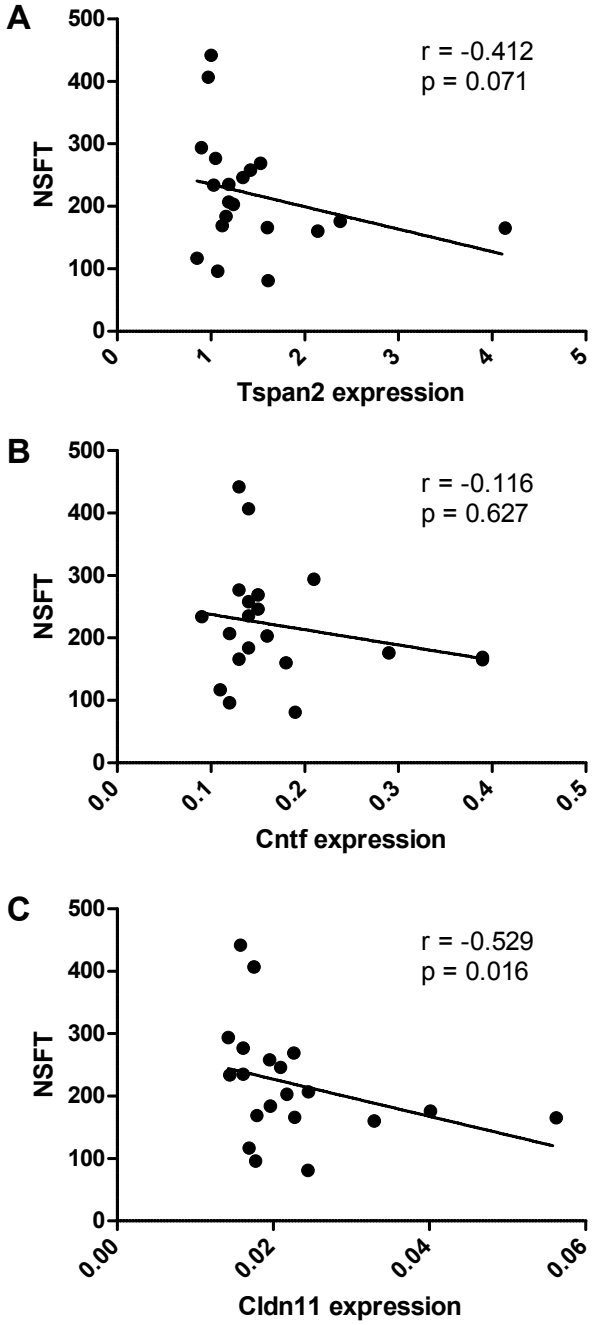
RNA-seq validation was performed by RT-qPCR analysis of selected genes using GoTaq® qPCR Master Mix (Promega Benelux b.v. Leiden, The Netherlands). Primers were designed using Primer3 online software (http://frodo.wi.mit.edu). See Supplementary Table 1 for primer sequences. Complementary DNA (cDNA) was synthesized using 1 µg of total RNA in a reverse transcription reaction using iScript cDNA Synthesis Kit according to manufacturer's protocol (Bio-Rad). qPCR reactions were performed in a 7500 Fast Real Time PCR System (Applied Biosystems, Foster City, CA, U.S.A) using the SYBR Green fluorescence quantification system (GoTaq® qPCR Master Mix, Promega). Thermal cycling was initiated with incubation at 95°C for 10 min followed by 40 cycles of 95°C for 30 sec and 60°C for 1 min. To normalize the cDNA content of the samples, we used the comparative threshold cycle (CT) method[2], which consists of the normalization of the number of target gene copies versus two endogenous reference genes *Ywhaz* and *Hprt1*.

Supplementary Table 1. Primers used for quantitative PCR

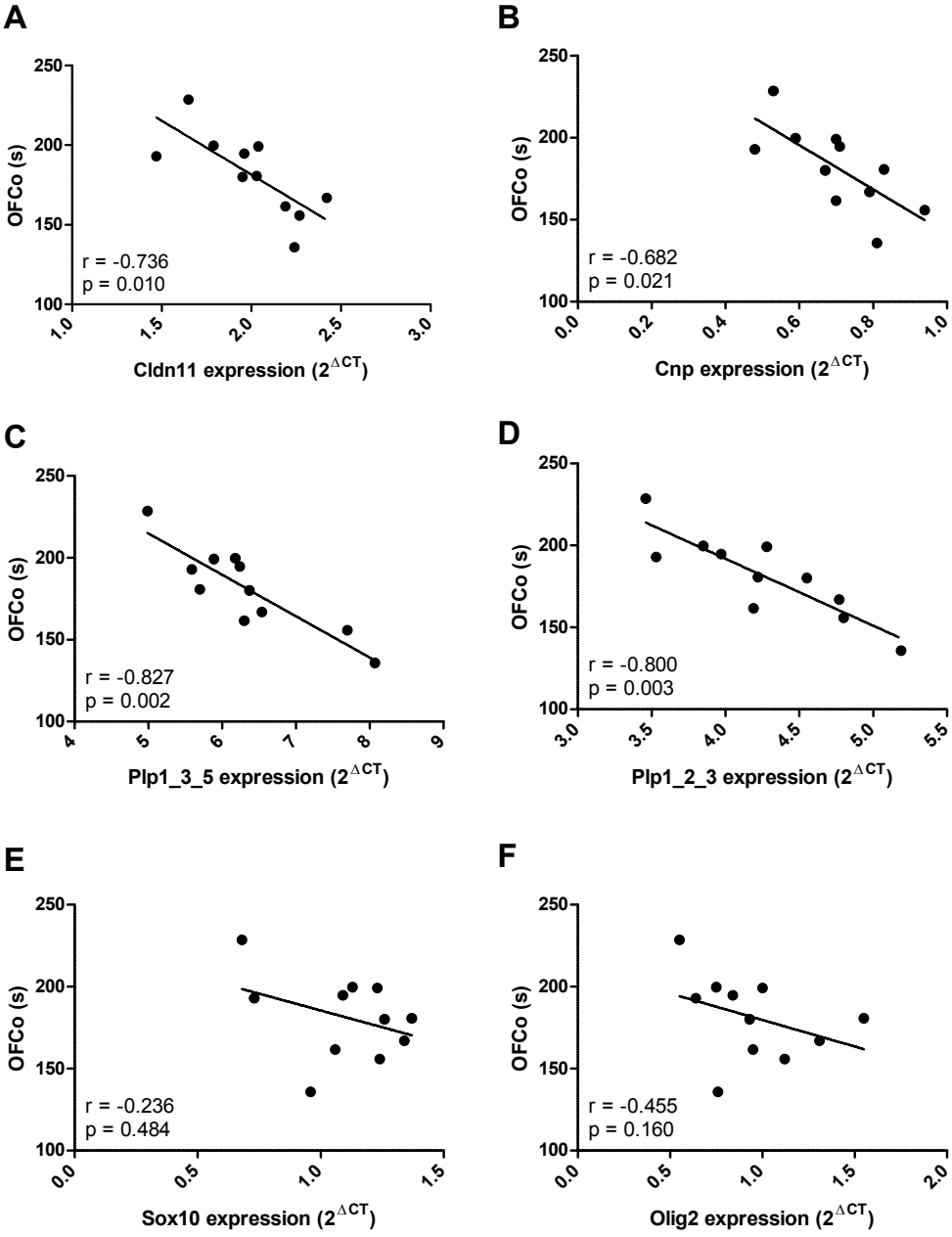
Gene symbol	Ref-seq number	Forward primer	Reverse primer
Olfm1	NM_053573.1	AGACCTCAGGCTCAAGGTTC	CACCATGGACTTGTACTCACG
Grina	NM_153308.4	AGGCTCTTCTGCGTCTTCC	AACTCTTTTCATGGGACATGG
Syn2	NM_001034020.1	ATGCGGATGGAACCTACG	GGATGAGCACGAAGTCTGG
Adcy1	NM_001107239.1	CTGTGTGGAGATGGGACTTG	CACACGCATGTTCAGGTCTAC
Src	NM_031977.1	GGAATCAGAGCGGCTACTTC	TTTCACATTTAGGCCCTTGG
Tspan2	NM_022589.1	CAGCTCATTGGAATTGTTGG	AGTTCCGTATTGCACAGCAG
Prkcd	NM_133307.1	GCCTTTGTCCTGAATGTGG	CCTTCCTCACCCATCTCATC
Cldn11	NM_053457.2	CGCATACAGGAAACCAGATG	CTGGGGTGCTCCTTATTCTG
Cpne4	NM_001109003.1	TCATCCTCAAGATGCAATCC	CCACCGTAAACAGCTTTGAG
Nts	NM_001102381.1	CTGCTTGTCAGAAGGCTGAG	GATCTGCCTCCAGGACTCTC
Sult5a1	NM_001201369.1	CAGAGTCACCCATCTTGGAC	ACCAGAGTCAGGGCAAGTTC
Cntf	NM_013166.1	CTTGCCACTGGTACACCATC	TCGTTCAGACCTGACTGCTC
Egr2	NM_053633.1	TGCCCATGTAAGTGAAGGTC	TGATCAGATGAACGGAGTGG
Igf1	NM_001082479.1	AAAGTCAGCTCGTTCCATCC	TCTTGTTTCCTGCACTTCCTC
Plp1 (ex2-3)	NM_030990.2	TCTCCAAAAACTACCAGGACTATG	GGCCCCATAAAGGAAGAAG
Plp1 (ex3-5)	NM_030990.2	TTTGGGAAAATGGCTAGGAC	TGCAGATGGACAGAAGGTTG
Olig2	NM_001100557.1	TCACAGGAGGAACCGTGTC	TGCTGGAGGAAGATGACTTG
Sox10	NM_019193.2	TCTTTGGGGTGGTTGGAG	GCTGCTATCCAGGCTCACTAC
Mag	NM_017190.4	AGACAATGGCAATCAGGATG	TTTGTACCTCCAGGAACCTCTAC
Tf	NM_001013110.1	GGAAAGTGCAGGCTTCTAGG	CAGAGATGACACCAAGTGTTTG
Mog	NM_022668.2	GAGGTTCTCGGATGAAGGAG	CAGGGTTGATCCAGTAGAAGG
Cnp	NM_012809.2	GGCAGAAGAATATGCCCAAC	TCACAAAGAGGGCAGAGATG
Hprt1	NM_012583.2	GCAGACTTTGCTTTCCTTGG	CGAGAGGTCCTTTTCACCAG
Ywhaz	NM_013011.3	TTGAGCAGAAGACGGAAGGT	GAAGCATTGGGGATCAAGAA

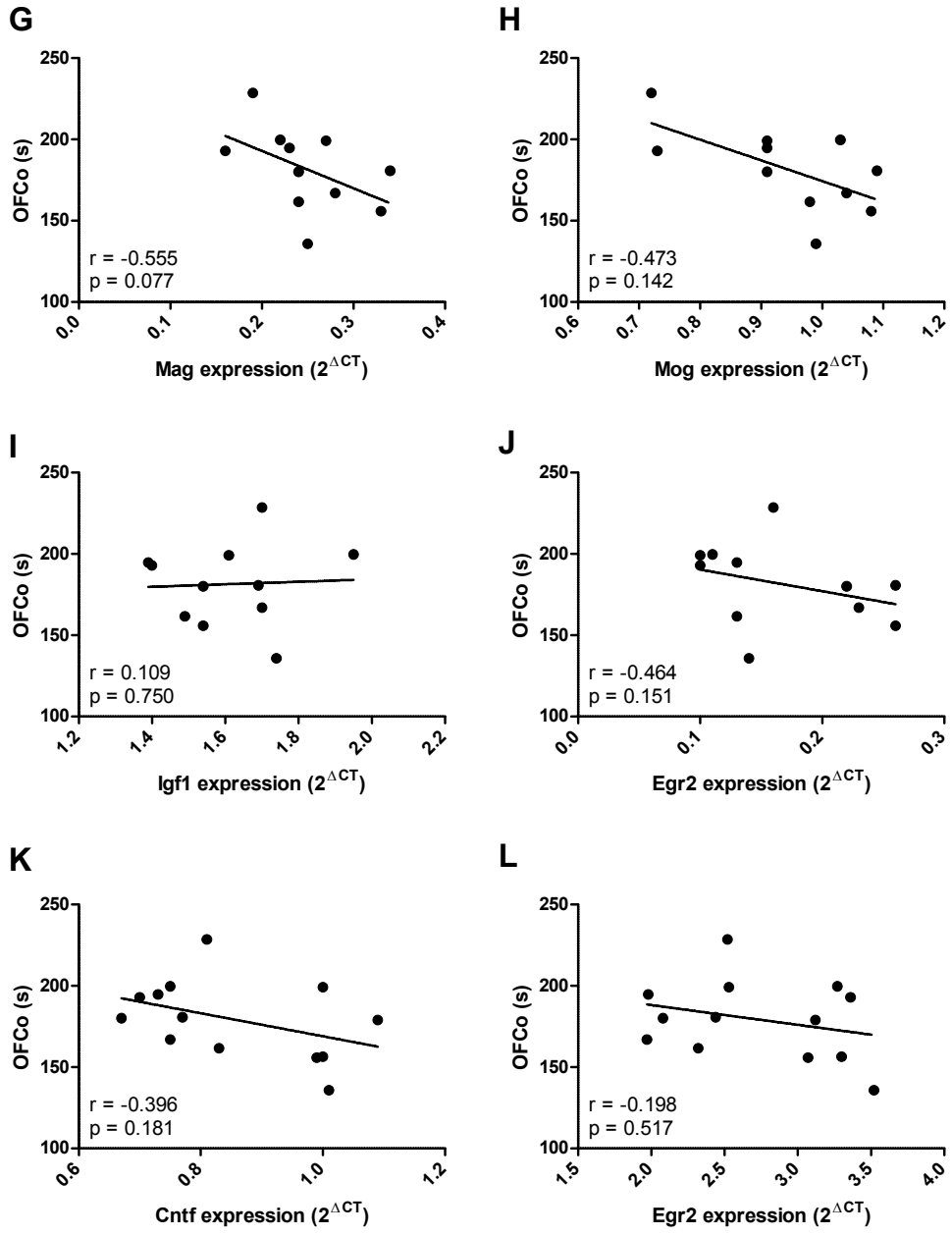
Supplementary Table 2. Body weight and novelty suppressed feeding test

Rat nr	Treatment	NSF Latency (s)	BW (g) 1	BW (g) 2	BW (g) 3	BW (g) 4	BW (g) 5	BW (g) 6	BW (g) 7	BW (g) 8	BW (g) 9	BW (g) 10	BW (g) 11	BW (g) 12	BW (g) 13	BW (g) 14	BW (g) 15	BW (g) 16	BW (g) 17	BW (g) 18	BW (g) 19	BW (g) 20	BW (g) 21
54	Vehicle	407	288	287	288	293	300	300	308	305	310	308	316	314	322	323	325	329	335	337	334	344	338
55	Vehicle	277	296	294	299	302	307	305	317	314	317	317	320	320	322	330	333	334	342	338	342	345	345
56	Vehicle	258	301	301	303	305	309	314	319	322	320	317	330	333	332	339	340	345	347	351	353	357	355
57	Vehicle	235	297	297	300	303	306	307	315	311	315	314	318	323	329	327	328	336	342	338	348	342	348
58	Vehicle	184	300	300	304	311	315	313	323	318	324	324	328	335	334	342	346	345	351	355	351	356	359
59	Vehicle	442	300	295	299	307	313	313	321	320	322	323	328	327	331	332	341	337	346	346	349	352	353
60	Vehicle		292	294	297	302	304	306	314	314	314	320	321	325	333	329	332	335	341	343	346	349	348
61	Vehicle	246	295	299	301	303	304	307	317	316	320	318	324	328	329	332	334	341	342	342	346	344	352
62	Vehicle	96	294	300	302	311	316	315	322	325	328	324	328	331	336	340	341	343	346	343	351	346	354
63	Vehicle		290	290	296	297	306	306	313	311	313	317	322	321	324	327	331	335	336	339	337	342	348
64	Vehicle	234	293	301	304	309	312	312	319	320	319	325	325	330	335	337	341	341	345	348	353	353	352
65	Vehicle	269	300	301	305	310	316	319	321	327	324	330	333	337	342	341	345	345	348	348	351	355	352
66	Fluoxetine	81	290	289	294	300	298	299	305	305	304	307	305	308	315	315	315	319	320	320	319	326	322
67	Fluoxetine	176	300	299	298	300	299	299	305	302	304	300	307	308	310	312	315	322	319	327	324	317	333
68	Fluoxetine	169	305	300	302	300	299	304	302	305	299	303	302	305	304	306	306	307	311	313	311	314	315
69	Fluoxetine	165	300	290	298	295	299	295	301	298	300	300	308	305	303	307	308	306	317	313	312	314	315
70	Fluoxetine	207	303	300	302	305	307	308	312	317	310	316	322	323	323	329	328	331	332	339	336	337	342
71	Fluoxetine	203	293	285	293	290	294	294	300	302	299	302	306	309	310	307	317	321	320	326	327	328	325
72	Fluoxetine		305	302	299	307	313	307	316	314	310	317	316	307	324	321	323	323	328	329	330	332	322
73	Fluoxetine	166	283	279	282	283	287	285	290	287	284	288	285	290	290	289	290	290	294	295	294	297	300
74	Fluoxetine	160	294	290	289	293	291	293	295	292	294	295	295	298	300	300	305	307	307	309	310	311	313
75	Fluoxetine	117	284	283	280	285	288	290	292	293	292	295	297	295	298	302	305	307	307	312	314	314	314
76	Fluoxetine	267	292	294	296	300	300	300	306	307	303	309	307	312	312	317	320	314	320	323	322	324	331
77	Fluoxetine	294	289	287	288	290	291	297	296	294	291	296	297	299	290	302	306	301	308	306	307	307	310

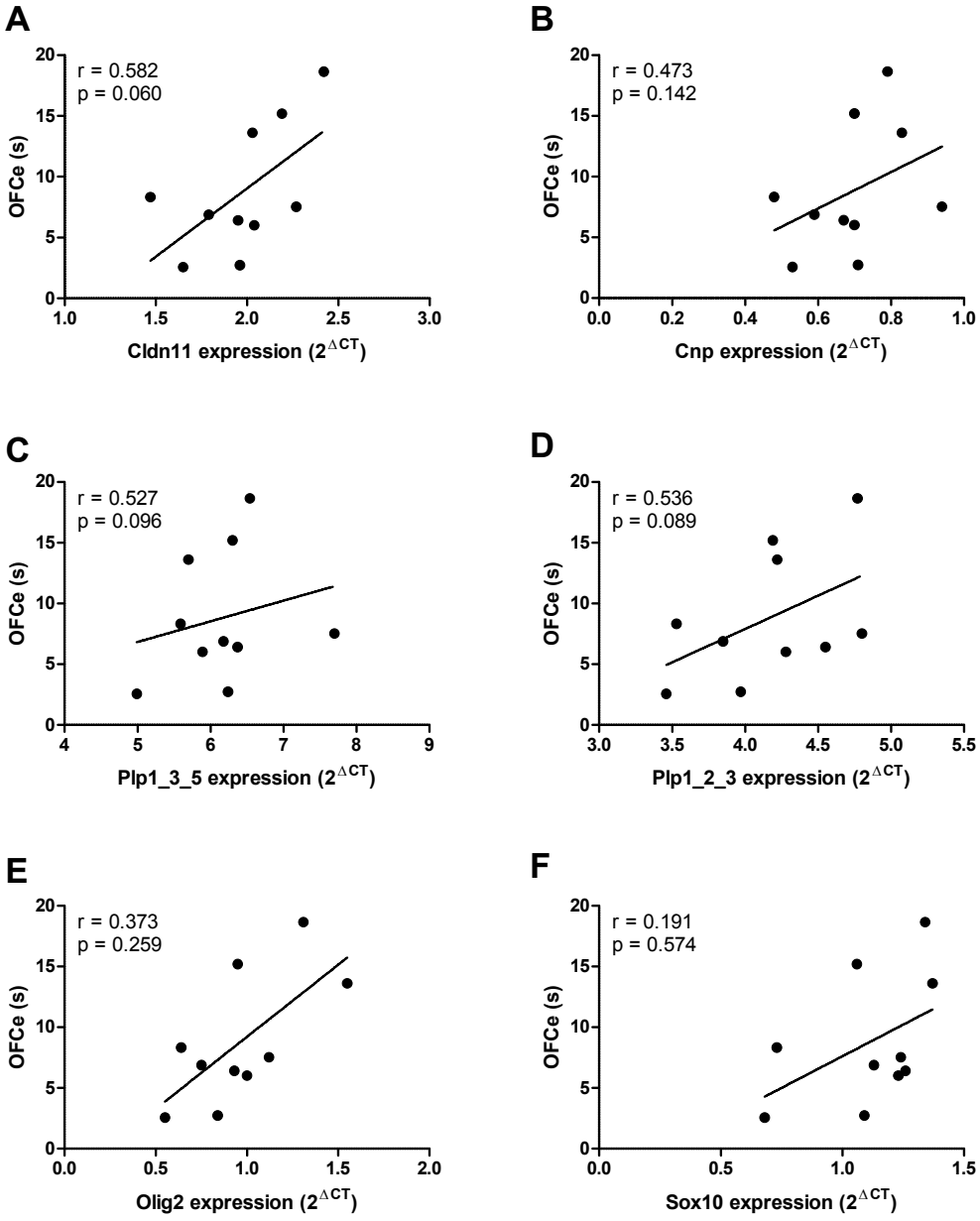


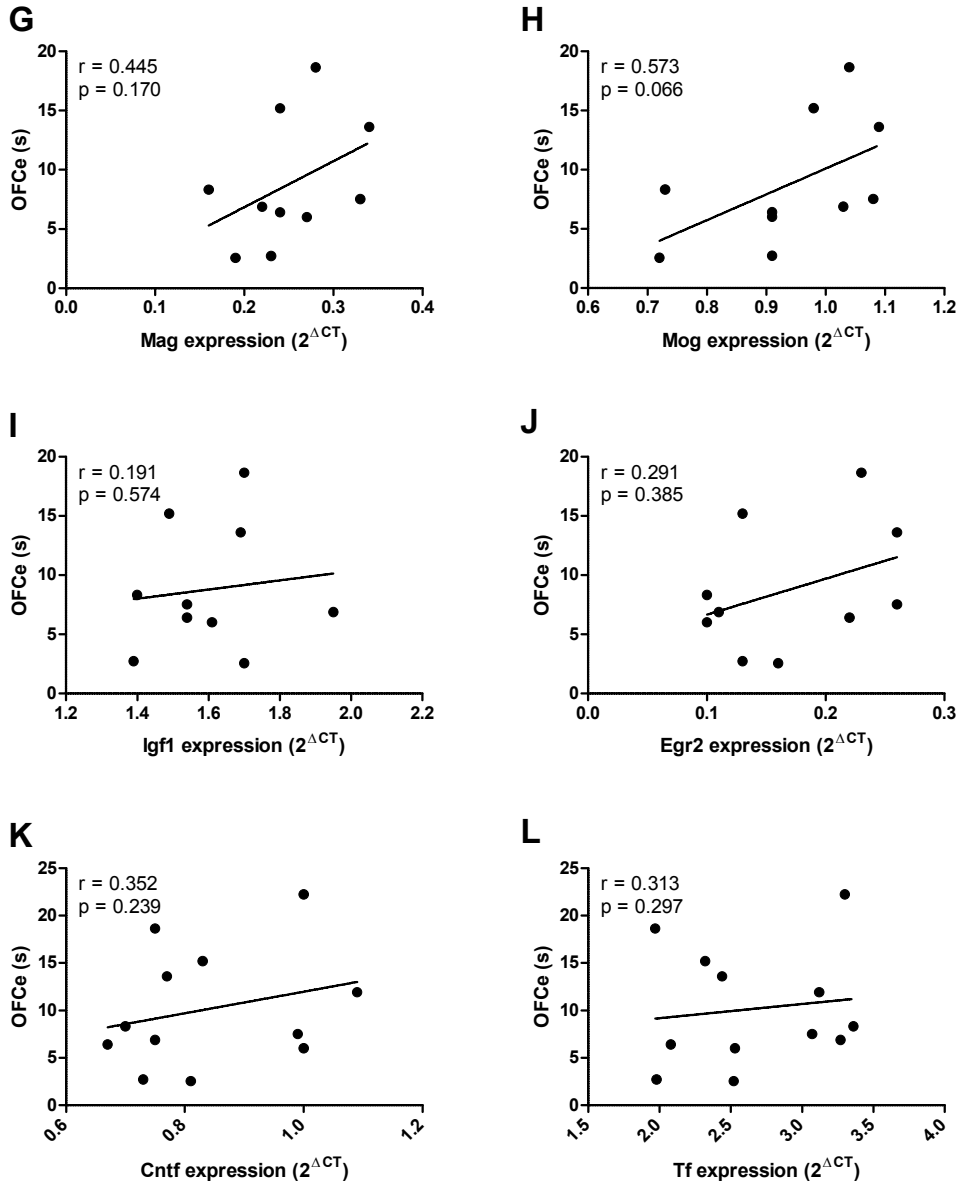
Supplementary Figure 1. Correlations between latency to start eating in the novelty suppressed feeding test (NSFT) and expression of myelination-related genes in adult fluoxetine-exposed rats. Spearman correlations between latency to start eating (s) and expression of myelination-related genes ($2^{\Delta CT}$). A significant negative correlation was found for latency to start eating and expression of *Cldn11* ($p<0.05$) and a trend for a negative correlation was found for latency to start eating and expression of *Tspan2* ($p<0.1$).


A
OFCo (s)
r = -0.736
p = 0.010
Cldn11 expression (2^ΔCT)
B
OFCo (s)
r = -0.682
p = 0.021
Cnp expression (2^ΔCT)
C
OFCo (s)
r = -0.827
p = 0.002
Plp1_3_5 expression (2^ΔCT)
D
OFCo (s)
r = -0.800
p = 0.003
Plp1_2_3 expression (2^ΔCT)
E
OFCo (s)
r = -0.236
p = 0.484
Sox10 expression (2^ΔCT)
F
OFCo (s)
r = -0.455
p = 0.160
Olig2 expression (2^ΔCT)

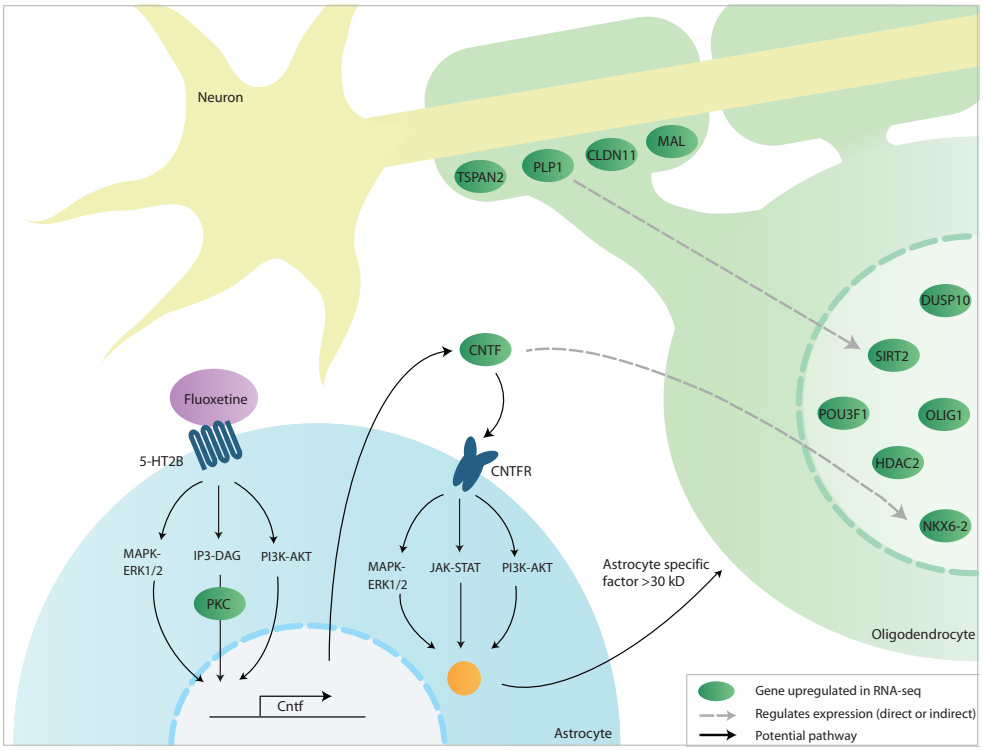


Supplementary Figure 2. Correlations between time spent in the corner (OFCo) and expression of myelination-related genes in neonatally fluoxetine-exposed rats. Spearman correlations between OFCo (s) and expression of myelination-related genes (2ΔCT). A significant negative correlation was found for OFCo and expression of Cldn11, Cnp, Plp1_3_5 and Plp1_2_3 ($p<0.05$) and a trend for a negative correlation was found for Mag ($p<0.1$).


A
r = 0.582
p = 0.060
OFCe (s)
Cldn11 expression (2^ΔCT)
B
r = 0.473
p = 0.142
OFCe (s)
Cnp expression (2^ΔCT)
C
r = 0.527
p = 0.096
OFCe (s)
Plp1_3_5 expression (2^ΔCT)
D
r = 0.536
p = 0.089
OFCe (s)
Plp1_2_3 expression (2^ΔCT)
E
r = 0.373
p = 0.259
OFCe (s)
Olig2 expression (2^ΔCT)
F
r = 0.191
p = 0.574
OFCe (s)
Sox10 expression (2^ΔCT)



Supplementary Figure 3. Correlations between time spent in the center (OFCe) and expression of myelination-related genes in neonatally fluoxetine-exposed rats. Spearman correlations between OFCe (s) and expression of myelination-related genes ($2^{\Delta CT}$). A trend for a positive correlation was found for OFCe and expression of *Cldn11, Plp1_3_5*, *Plp1_2_3* and *Mog* ($p<0.1$).



Supplementary Figure 4. Potential pathway for fluoxetine affecting myelination-related genes. It has been shown that fluoxetine can stimulate the 5-HT_{2B} receptor on astrocytes, which results in activation of its downstream signaling cascades[3]. The 5-HT_{2B} receptor is $G_{q/11}$ protein coupled and stimulates the diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3) pathway (IP3-DAG). DAG is the physiological activator of protein kinase C (PKC), which in turn can activate the mitogen-activated protein kinase (originally called ERK, extracellular signal-regulated kinase) pathway (MAPK-ERK1/2)[3]. A third signal transduction pathway activated by fluoxetine-induced stimulation of the 5-HT_{2B} receptor is the PI3K-AKT pathway[3]. Activation of these signal transduction pathways results in transcription factor activation and transcription of neurotrophic factors. MAPK-ERK1/2 activation by fluoxetine results in transcription of glial-derived nerve factor (GDNF)[3, 4]. In addition, transcription of brain derived neurotrophic factor is induced, but not through the MAPK-ERK1/2 pathway[4]. Possibly also transcription and release of ciliary neurotrophic factor (CNTF) is triggered by fluoxetine. The released CNTF can bind to the CNTF receptor triggering intracellular signaling through three major signal transduction pathways: JAK- STAT, MAPK-ERK1/2 and PI3K-AKT[5]. These signal transduction pathways all mediate different responses. Studies have shown that CNTF-activated astrocytes release an astrocyte specific factor (>30 kD), which promotes proliferation and survival of oligodendrocyte precursor cells[6]. Furthermore, studies have shown that CNTF can induce maturation of oligodendrocytes[7, 8]. An increase in the number of oligodendrocyte precursors and mature oligodendrocytes in our hippocampus tissue might explain the enhanced expression of myelination-related genes. It has been shown that the expression of NKX6.2 in oligodendrocytes is strongly induced after CNTF treatment (grey dotted line)[9] and that PLP1 is required for transport and expression of SIRT2 in myelin (grey dotted line)[10].

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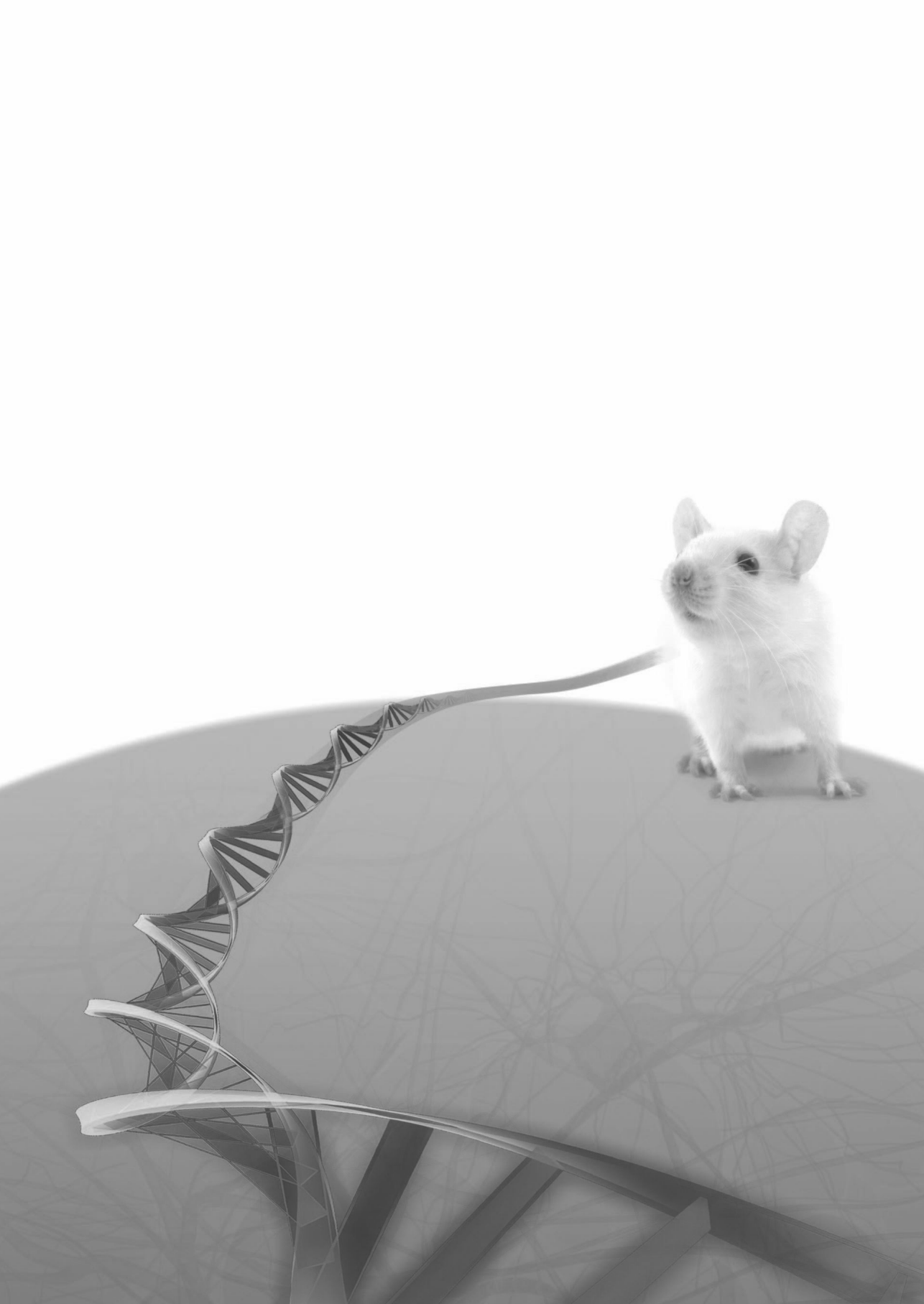

4

Perinatal reduction of functional serotonin transporters results in developmental delay

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Abstract

While there is strong evidence from rodent and human studies that a reduction in serotonin transporter (5-HTT) function in early-life can increase the risk for several neuropsychiatric disorders in adulthood, the effects of reduced 5-HTT function on behavior across developmental stages are underinvestigated. To elucidate how perinatal pharmacological and lifelong genetic inactivation of the 5-HTT affects behavior across development, we conducted a battery of behavioral tests in rats perinatally exposed to fluoxetine or vehicle and in 5-HTT$^{-/-}$ versus 5-HTT$^{+/+}$ rats. We measured motor-related behavior, olfactory function, grooming behavior, sensorimotor gating, object directed behavior and novel object recognition in the first three postnatal weeks and if possible the tests were repeated in adolescence and adulthood. We also measured developmental milestones such as eye opening, reflex development and body weight. We observed that both pharmacological and genetic inactivation of 5-HTT resulted in a developmental delay. Except for hypo-locomotion, most of the observed early-life effects were normalized later in life. In adolescence and adulthood we observed object directed behavior and decreased novel object recognition in the 5-HTT$^{-/-}$ rats, which might be related to the lifelong inactivation of 5-HTT. Together, these data provide an important contribution to the understanding of the effects of perinatal and lifelong 5-HTT inactivation on behavior across developmental stages.

Introduction

Serotonin (5-hydroxytryptamine; 5-HT) is an important neurotransmitter in the central nervous system, where it has various functions including the regulation of mood[1], learning and memory[2,3], appetite[4] and sleep[5,6]. 5-HT is produced by serotonergic neurons in the raphe nuclei, which send their axons to regions throughout the brain. Serotonin transporters (5-HTT) on serotonergic neurons facilitate the reuptake of extracellular 5-HT in the serotonergic neuron and thereby regulate extracellular 5-HT levels. In the adult brain 5-HTT is only expressed in the raphe neurons, but at early developmental stages (embryonic day 11 to postnatal day (PND)13 in rodents[7]) 5-HTT expression pattern is more widespread. The functional implication of the transient 5-HTT expression is not completely clear, but the idea is that it may serve to maintain stable 5-HT levels important for developmental processes. Hence, during neurodevelopment 5-HT acts not only as a neurotransmitter but also as a neurotrophic factor. Specifically, during early brain development 5-HT can promote neurodevelopmental processes like neuronal outgrowth, synaptogenesis and migration processes[8-12].

Alterations in the serotonergic system are involved in a variety of affective disorders, with depression as the most well-studied disorders in relation to 5-HT[13,14]. Most of the drugs prescribed for treatment of depression act on the serotonergic system and increase extracellular 5-HT levels to treat depression-like symptoms. Selective serotonin reuptake inhibitors (SSRIs) are the most frequently prescribed drugs for treatment of depression and act by blocking the serotonin transporter, resulting in increased levels of extracellular serotonin[15-17]. Besides their function as antidepressant, SSRIs are also prescribed for the treatment of anxiety-related disorders[18,19] and autism spectrum disorders[20,21]. While a lot of studies focused on the 'positive' effects of increasing extracellular 5-HT levels in the mature brain, evidence is accumulating that increasing extracellular 5-HT levels in the developing brain is associated with 'negative' behavioral outcomes, resembling phenotypes of neuropsychiatric disorders.

Pregnancy is a period with increased risk for depression. Accordingly, there is a substantial number of women taking SSRIs (prevalence between 7.4% and 14%[22,23]). It has been shown that antidepressants can pass from mother to child[24] by crossing the placenta[25] and via breast milk[26]. The most reported effects in children perinatally exposed to SSRIs are neonatal withdrawal symptoms, which include continuous crying, irritability, jitteriness and/or restlessness; shivering; fever; tremors; hypertonia or rigidity; respiratory distress; feeding difficulty; sleep disturbance; hypoglycemia; and seizures [27]. The onset of these symptoms ranges from several hours to several days after birth and the symptoms are usually resolved within 2 weeks[27]. In addition, a few human studies showed teratogenic effects, like an increased risk for preterm birth[28], low birth weight[23,29] as well as persistent pulmonary hypertension (PPHN)[30,31], congenital heart defects[32-35] and other congenital malformations[33] in the newborn. Given that serotonin is important for brain development, it is likely that also early-life behavior is affected after perinatal SSRI exposure. Indeed, there are a few human studies reporting developmental delay associated with 2nd and 3th trimester SSRI use, which in

most studies reflects a delay in gross motor function milestones (e.g. sitting and walking)[36-38]. Studies investigating longer term effects in children (up to 7 years of age) suggest persistent effects on motor function[39] and language development[40,41]. Furthermore, there is evidence that perinatal SSRI exposure increases the likelihood of symptoms related to autism spectrum disorders[42-44] in the offspring. However, there are also studies showing no effect of perinatal SSRI exposure on birth weight[45,46], congenital malformations[47-49] and neurodevelopmental behavior[50,51].

A limitation of most human studies is that the type of SSRI, exposure period and dose are often mixed in this studies. Furthermore, environmental factors (including depression of the mother) might influence the development and behavior of the child, making long-term studies (up to adulthood) in humans challenging. To remedy this problem researchers use rodents to study the effects of developmental SSRI exposure in a more controlled environment and to focus on a specific SSRI type, dose and SSRI exposure period. In rodents treated with SSRIs during pregnancy also teratogenic effects have been shown, like a shortened gestational length[52], delivery of smaller pups[52,53], less weight gain in the pups[53] and heart failure[32]. At the behavioral level most rodent studies focused on anxiety and depression-like behavior and showed that there is ample evidence that perinatal exposure to SSRIs results in 'paradoxical' anxiety- and depression-like symptoms in adulthood[54-56]. However, there are also studies showing that anxiety-like behavior can be decreased in adulthood after early-life SSRI exposure[57]. In addition, there are a few studies showing early-life behavioral changes in perinatally SSRI-exposed rodents. Deiro and colleagues studied reflex development in rats exposed to SSRIs (sertraline or citalopram) during early-postnatal development and found a delay in development of several reflexes[58,59]. Also two studies using fluoxetine showed some early-life effects of perinatal SSRI exposure on reflex development[60] and muscle strength[61]. There are a few studies in rodents showing long-term effects up to adulthood. The most common long-term findings were (sensori)motor-related defects in adolescence[62,63] and decreased locomotor activity[55,64] and a reduction in sexual behavior in adulthood[65-67]. Furthermore, there is evidence that perinatal SSRI exposure increases the likelihood of symptoms related to autism spectrum disorders[44,61,68].

Another example of an alteration in the serotonergic system already present in early-life is a variation in the promoter region of the serotonin transporter gene (*SLC6A4*). The short allelic variant (S-allele) of the serotonin transporter promoter-linked polymorphic region (5-HTTLPR), is associated with reduced 5-HTT protein availability and function[69] compared with the long (L) form. S-allele carriers have an increased risk for developing a variety of neuropsychiatric disorders, like depression[70], anxiety[71] and drug addiction[72]. To model life-long impairment of 5-HTT protein availability and function in rodents (as in the S-allele carriers) a 5-HTT knockout (5-HTT$^{-/-}$) rat has been created[73]. These 5-HTT$^{-/-}$ rats also display increased anxiety[74] and depression-like behavior[74] and compulsive cocaine self-administration behavior[72]. Despite the lifelong increase in extracellular 5-HT levels in the 5-HTT$^{-/-}$ rats, the phenotype resembles the phenotype seen after perinatal SSRI exposure,

suggesting that the 5-HTT$^{-/-}$ phenotype is mainly caused by increased 5-HT levels in early-life. To the best of our knowledge there are no studies that have investigated behavior across developmental stages in S-allele carriers or in 5-HTT$^{-/-}$ rats. In sum, early-life inactivation of functional 5-HTT, by blocking 5-HTT using SSRIs or by genetic reduction of 5-HTT, leads in adulthood to phenotypes that are part of a variety of neuropsychiatric disorders. Whereas SSRI studies revealed that perinatal 5-HTT inactivation can have detrimental effects on early-life behavior, with motor deficits as the most common finding, this remains to be studied for genetic 5-HTT inactivation conditions. Furthermore, it is unknown how behavior changes over developmental stages in individuals with perinatal or life-long 5-HTT inactivation. As the effects seen in rats with reduced 5-HTT function resemble the effects observed in humans with reduced 5-HTT function (e.g. depression, anxiety, autism and motor coordination), these rats are a good model for translational research.

To elucidate how perinatal pharmacological and life-long genetic inactivation of the 5-HTT affects behavior across development, we conducted a battery of behavioral tests in rats perinatally exposed to fluoxetine or vehicle and in 5-HTT$^{-/-}$ versus 5-HTT$^{+/+}$ rats. We performed tests for reflex development, motor-related behavior, olfactory function, grooming behavior, sensorimotor gating, object directed behavior and novel object recognition. In addition, we measured body weight and eye opening. Behavioral tests were performed in the first three postnatal weeks and several tests were repeated in adolescence and adulthood to see whether the early-life effects have long-term consequences.

Materials and methods

Animals

For perinatal fluoxetine and vehicle treatment, female Wistar rats were obtained from Harlan (Horst, The Netherlands) and housed in standard Macrolon® type 3 cages (42x26x20 cm, Nijmegen, Gelderland, The Netherlands) in temperature-controlled rooms (21 °C ± 1 °C) under standard 12-h light/dark cycle (lights on at 7:00 A.M.) with food (Sniff, long cut pellet, Bio Services, Uden, The Netherlands) and water available *ad libitum*. To assess the estrous stage, the cycle of female Wistar rats was measured daily in the late afternoon with an impedance checker (Impedance Checker MK-10B, Muromachi Kikai, Tokyo, Japan). When a female reached the estrous stage it was placed together with a male in a Macrolon type 3 cage (42×26×20 cm) with a wire gauze bottom. The next day the cage was explored for a vaginal plug, and if found, this day was considered as gestational day (GD) 1. From GD11 until 7 days after delivery dams were treated daily with 12 mg/kg fluoxetine or 1% methylcellulose (vehicle). The rats were randomly assigned to a treatment group. Pups were weaned at PND22. Three vehicle (n=16) and three fluoxetine-exposed (n=12) nests were used for experiments in early-life, adolescence and adulthood. Only the male pups were used for experiments and the following experiments were performed: body weight, eye opening, righting reflex, walking, swimming, negative geotaxis, vibrissa placing, bar holding, object directed behavior (ODB), novel object recognition (NOR), olfactory discrimination, grooming,

startle reflex and prepulse inhibition (PPI).

5-HTT$^{-/-}$ rats (Slc6a41Hubr) were generated by ENU-induced mutagenesis[75]. Rats were housed in standard Macrolon® type 3 cages in temperature-controlled rooms (21 °C ± 1 °C) under standard 12-h light/dark cycle (lights on at 7:00 A.M.) with food (Sniff, long cut pellet, Bio Services) and water available *ad libitum*. For experimental group 1, four 5-HTT$^{+/+}$ (n=21) and three 5-HTT$^{-/-}$ (n=16) nests were used and the following experiments were performed: Body weight, eye opening, righting reflex, walking, swimming, negative geotaxis, vibrissa placing, olfactory discrimination and grooming. For experimental group 2, three 5-HTT$^{+/+}$ (n=19) and three 5-HTT$^{-/-}$ (n=17) nests were used for experiments. The tests that were performed are body weight, bar holding, ODB, startle reflex and PPI. A third experimental group (group 3) was used for testing behavior in adolescence and in adulthood. Three 5-HTT$^{+/+}$ (n=11) and three 5-HTT$^{-/-}$ (n=12) nests were used for body weight, grooming, ODB, NOR, startle reflex and PPI. In addition, a fourth group was used for measuring brain weight. Male rats were weighted and sacrificed by decapitation at four different time points, PND8, PND14, PND21 and PND35. Brains were collected and brain weight was measured. For each time point three 5-HTT$^{+/+}$ and three 5-HTT$^{-/-}$ nests were used. Brain material was used for molecular experiments (not in this paper). See Supplementary Figure 1 for the timetable of all groups. The investigator was not blinded to the group allocations when performing the experiments. Tests based on scoring (swimming, walking and vibrissa placing) were reanalyzed by a second researcher (blinded). All efforts were made to minimize animal suffering and to reduce the number of animals used. All experiments were carried out according to the guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council 2003), the principles of laboratory animal care, as well as the Dutch law concerning animal welfare.

Drug treatment

Dams received fluoxetine (12 mg/kg/day, as used by Olivier and colleagues[54]) or vehicle by oral gavage (every morning) from GD11 to PND7 in a volume of 5 ml/kg. Fluoxetine was purchased from the Pharmacy of the Radboud University Nijmegen Medical Centre, The Netherlands and dissolved in distilled water. As a vehicle, 1% methylcellulose (Genfarma B.V. Maarssen, The Netherlands) was used, which was the constituent of the fluoxetine pills.

Early postnatal morphological development

Body weight of the rats was measured daily from PND2 to PND21 and on PND35 and PND70. Starting from PND13 eye opening was checked in the morning and in the afternoon. The rats scored (0) when both eyes were closed, (1) when one eye was open and (2) when both eyes were open. For measuring brain weight, rats were sacrificed on PND8, 14, 21 and 35. After collecting the brain, the weight of the brain was measured and corrected for body weight (brain/body mass index) which was measured right before decapitation.

Reflex testing

Four tests were used for investigating reflex development. Righting reflex was tested from PND2 to PND10. The rats were placed on its back and the time (in sec) till righting (turning to its belly) was measured. Negative geotaxis reflex was tested from PND4 to PND14. The rat was placed on a tilted surface (40°) with its head facing downwards and the time (in sec) till turning 180° (head facing upwards) was measured. After each rat the arena was cleaned with ethanol (70%) and dried thoroughly to prevent transmission of olfactory cues. Vibrissa placing was tested from PND6 to PND14. Vibrissa placing was tested as described by Deiro and colleagues[58]. In short, the rat was held by the tail, the head facing the edge of the table and vibrissae just touching the vertical surface. The rats showed the vibrissa reflex when it lifted its head and extended the fore limbs in the direction of the table. The scores were defined as follows: (1) when showing the reflex and (0) when the reflex was absent. Startle reflex was tested on PND21, 35 and 69, method see below (section acoustic startle reflex and prepulse inhibition).

Muscular strength and motor coordination

Swimming ability was tested by dropping the rats in a water tank from a height of around 20 cm. The temperature of the water was 27°C (±1°C). The rat's swimming behavior was scored as described by Schapiro and colleagues[76] with small adaptations: (1) head is fully under water; (2) back of the head is above the water's surface, but ears are still partially under water and nose is pointing downwards; (3) nose above the water's surface, but ears still partially under water or (4) entire head remains above the water's surface (see Supplementary Figure 2). The experiment was performed daily between PND8 and PND14. Bar holding was tested daily from PND10 to PND19 and on PND21. Rats were placed with the forepaws on a wooden bar (3 mm or 5 mm in diameter, 40 cm long, 45 cm above the bench surface) and the time (in sec) till falling from the bar was scored (adapted from Deacon[77]). If the rat touched one of the end columns with one forepaw it obtained the maximum time of 50 sec. Unstable walking was tested from PND3 to PND14 by placing a rat on a coarse surface for maximal 30 seconds. Shaking and unstable walking was noted as 'present' if it occurred at least once in this 30 seconds. Scoring was as follows: (1) Shaking and unstable walking present, (0) no unstable walking and shaking, as described before by Balemans and colleagues[78].

Olfactory discrimination

Olfactory discrimination was tested on PND8, 10 and 12. Each rat was placed in the center of an empty cage in a neutral position. On one side of the cage, fresh sawdust was placed, while on the other side bedding from the homecage (including several pieces of faeces) was placed. The side of the fresh sawdust was changed every day, to prevent bias caused by environmental cues. The time the rat needed to reach the bedding from its homecage (with their mothers odour) was measured. The experiment ended when the rat reached the bedding from its homecage or after 240 seconds. After each rat the arena was cleaned with

ethanol (70%) and dried thoroughly to prevent transmission of olfactory cues.

Grooming behavior

Grooming behavior was tested on PND14, 17, 21, 35 and 69. A rat was placed in a glass cylinder with a diameter of 25 cm and was given one minute to explore. After 1 min the rat was sprayed two times with water (+/- 27°C). The next five minutes grooming behavior was scored according to three properties: latency to grooming onset, total duration of grooming and total number of grooming episodes. This scoring method was adapted from Kalueff & Tuohimaa[79]. After each rat the arena was cleaned with ethanol (70%) and dried thoroughly to prevent transmission of olfactory cues.

Object directed behavior and novel object recognition

On PND20, 34 and 68 rats were tested for ODB and NOR. In the first trial three objects differing in color and shape were placed in the area (5 cm from the walls) and the rat was able to explore the objects for 7 minutes. Each rat was tested once and the same three objects were used for all rats counterbalanced for position. The time exploring each object was measured and ODB was calculated (exploration time most explored object/total exploration time[80]). After an intertrial interval of eight hours NOR was measured (in 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats only performed in adolescence and adulthood) by replacing the object that was explored most in trial 1 by a new object. The rat was able to explore the objects for 7 minutes. The time exploring each object was measured and discrimination index (d2) was calculated (exploration time new object/total exploration time). Different sets of objects were used for the different developmental time points. Using a tracking system (EthoVision, Noldus, Wageningen, The Netherlands) the total distance moved was measured in both tests. After each rat the area was cleaned with alcohol (70%).

Acoustic startle reflex and prepulse inhibition

On PND21, 35 and 69 startle reflex and prepulse inhibition experiments were performed in four acoustic startle chambers of San Diego Instruments (San Diego, USA) as described by Sontag and colleagues[81]. In short, rats were placed in a Plexiglas tube (PND21: 3.8 cm in diameter, 10.5 cm in length; PND35, 69: 8.2 cm in diameter, 25 cm in length) resting on a plastic frame. A piezoelectric accelerometer mounted under the tube detected and transduced the motion of the tube. Throughout the startle session, a background noise of 70 dB was maintained. The experiment started with a 5 min habituation session with background noise in the startle system. After this habituation period, ten blocks of five trials were delivered to measure prepulse inhibition. Each of these blocks consisted of one startle trial (120 dB, 20 ms broad band burst), one no-stimulus condition, and three different prepulse-startle pairings, administered pseudorandomly. In these pairings, the prepulse was 3, 5, or 10 dB above background and always 20 ms broadband burst, followed by the 120 dB startle pulse 100 ms later. The interval between two trials was between 10 and 20 s. The startle amplitude

was calculated as the mean of 10 delivered startle trials. The degree of prepulse inhibition (in percentage) was calculated according to the formula: 100 – (Average of startle amplitude on prepulse trial/ Average of startle amplitude on startle trial) × 100.

Statistical analyses

Statistical analysis of the data was carried out using the IBM Corp. Statistical Package for the Social Sciences (SPSS) version 20.0 (IBM Corp, Armonk, NY, USA). Repeated measures analysis of variance (ANOVA) was applied on continuous data. Data were further analyzed per day using independent samples *t* tests (corrected p value was used when equal variance was not assumed). Mann-Whitney U tests were used for non-normal distributions. A Fisher's exact test was performed on categorical data. For each behavior test p values were corrected for multiple testing (adjusted p value) using the Benjamini-Hochberg method[82]. Pearson (continuous data) and Spearman (for categorical and non-normal distributed data) correlations were performed for correlations between the different behavior tests. Outliers (data points further than 3 interquartile ranges from the nearer edge of the box plot) were excluded from the analysis. All tests were performed two-sided and the level of statistical significance was set at $p<0.05$ in all tests. The behavior in the 5-HTT$^{-/-}$ versus 5-HTT$^{+/+}$ rats was assessed in three different groups and therefore no repeated measures ANOVA could be applied to the complete timeline up to adulthood. Separate tests were performed for adolescent and adult behavior. For consistency in data analysis, the same statistical analysis was used for the fluoxetine- versus vehicle-exposed group. The 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats were not directly compared with the vehicle and fluoxetine-exposed rats since differences in handling (fixation and oral gavage, potentially causing stress) and differences in their genetic background (ordered by a company or own breeding) can affect development and behavior in the offspring[83,84].

Results

Effect of genetic and pharmacological 5-HTT inactivation on early postnatal morphological development

Body weight of the offspring was followed from PND2 to PND21. In the fluoxetine- and vehicle-exposed group, repeated measures ANOVA revealed a significant age effect ($F_{(1.28, 33.31)}$=2653.41; $p<0.05$) and a significant treatment effect ($F_{(1,26)}$=11.00; $p<0.05$), showing that early-life fluoxetine exposure significantly reduced body weight gain. Independent samples *t* tests indicated that the reduction in body weight gain was significant ($p<0.05$) from PND2 to PND14 and a trend was seen on PND15,16 and 21 ($p<0.1$). Body weight was also measured in adolescence (PND35) and adulthood (PND69). We found significantly reduced weights in the fluoxetine-exposed rats compared to vehicle-exposed rats (adolescence: $t_{(1,26)}$= 2.11; $p<0.05$, adulthood: $t_{(1,26)}$= 2.93; $p<0.05$) (Figure 1A). In 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats (group 1) repeated measures ANOVA revealed a significant age effect ($F_{(1.41,46.67)}$= 2234.31; $p<0.05$), genotype effect ($F_{(1,33)}$=4927.80; $p<0.05$) and age x genotype interaction ($F_{(1.41,46.67)}$=42.94; $p<0.05$).

Independent samples *t* tests indicated that the body weight of 5-HTT$^{-/-}$ rats was significantly reduced ($p<0.05$) compared to 5-HTT$^{+/+}$ rats for all testing days (group 2 revealed similar results, not shown). Body weight was also measured in adolescence (PND35) and adulthood (PND69) and no significant differences were found (group 3) (Figure 1B). Eye opening was scored and showed no significant difference for both the 5-HTT$^{-/-}$ rats (group 1) and the fluoxetine-exposed rats compared to their controls (Figure 1C and D). In 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats (group 4) brain weight was measured and corrected for body weight (brain/body mass index) on PND8, 14, 21 and 35. Independent samples *t* tests showed a significantly increased brain/body mass index in 5-HTT$^{-/-}$ rats compared to the 5-HTT$^{+/+}$ rats on PND8 ($t_{(1,27)}=4.369$; $p<0.05$) and PND14 ($t_{(1,27)}=5.110$; $p<0.05$) and a trend on PND35 ($t_{(1,2)}=6.525$; $p<0.1$) (Figure 2A). As shown for group 2 and 3, body weight was also reduced in the 5-HTT$^{-/-}$ rats of group 4 (data not shown). In addition, brain weight was significantly reduced in 5-HTT$^{-/-}$ rats compared to the 5-HTT$^{+/+}$ rats on PND8 ($t_{(1,28)}=2.43$; $p<0.05$), PND14 ($t_{(1,27)}=3.70$; $p<0.05$) and PND35 ($t_{(1,5)}=7.64$; $p<0.05$) (Figure 2B). Unfortunately, we did not measure brain weight of rats sacrificed at PND70 and we have to keep in mind that the results of PND35 are based on a low number of animals. Brain weight was not measured in perinatally fluoxetine- versus vehicle-exposed rats.

Effect of genetic and pharmacological 5-HTT inactivation on reflex development

Negative geotaxis was tested from PND4 to PND14. Rats perinatally exposed to fluoxetine or vehicle showed a significant age effect ($F_{(4.91,127.55)}=59.53$; $p<0.05$) and treatment effect ($F_{(1,26)}=10.86$;$p<0.05$) in the negative geotaxis test. Fluoxetine-exposed rats showed a significantly longer turning time compared to vehicle-exposed rats in repeated measures ANOVA, however independent samples *t* tests revealed no significant differences on separate days (Figure 3A). In the 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats (group 1) repeated measures ANOVA also showed a significant age effect ($F_{(10,320)}=57.60$; $p<0.05$), genotype effect ($F_{(1,32)} = 45.00$; $p<0.05$) and age x genotype interaction ($F_{(10,320)}=2.56$; $p<0.05$). Independent samples *t* tests demonstrated that 5-HTT$^{-/-}$ rats show significantly ($p < 0.05$) longer turning times in the geotaxis experiment compared to 5-HTT$^{+/+}$ rats on PND9 to PND12 (Figure 3B). In the vibrissa placing test (PND6 to PND14) perinatally fluoxetine-exposed rats showed a significant delay in developing the vibrissa placing reflex compared to vehicle-exposed rats on PND10 and PND11 ($p<0.05$) using a Fisher's exact test (Figure 3C). The 5-HTT$^{-/-}$ rats (group 1) showed a significant delay in reflex development compared to 5-HTT$^{+/+}$ rats on PND10 ($p<0.05$) using a Fisher's exact test (Figure 3D). Vibrissa placing videos were re-analyzed by a second researcher resulting in similar results (data not shown). The righting reflex was tested from PND2 to PND10. In the rats perinatally exposed to fluoxetine or vehicle repeated measures ANOVA revealed an age effect ($F_{(1.43,\ 28.69)}=18.99$; $p<0.05$), but no significant differences in turning time between the fluoxetine-exposed and vehicle-exposed rats (Figure 3E). In the 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats (group 1) repeated measures ANOVA demonstrated a significant age effect ($F_{(2.41,81.90)}=17.23$; $p<0.05$) and a trend for a significant genotype effect ($F_{(1,34)}=3.75$;

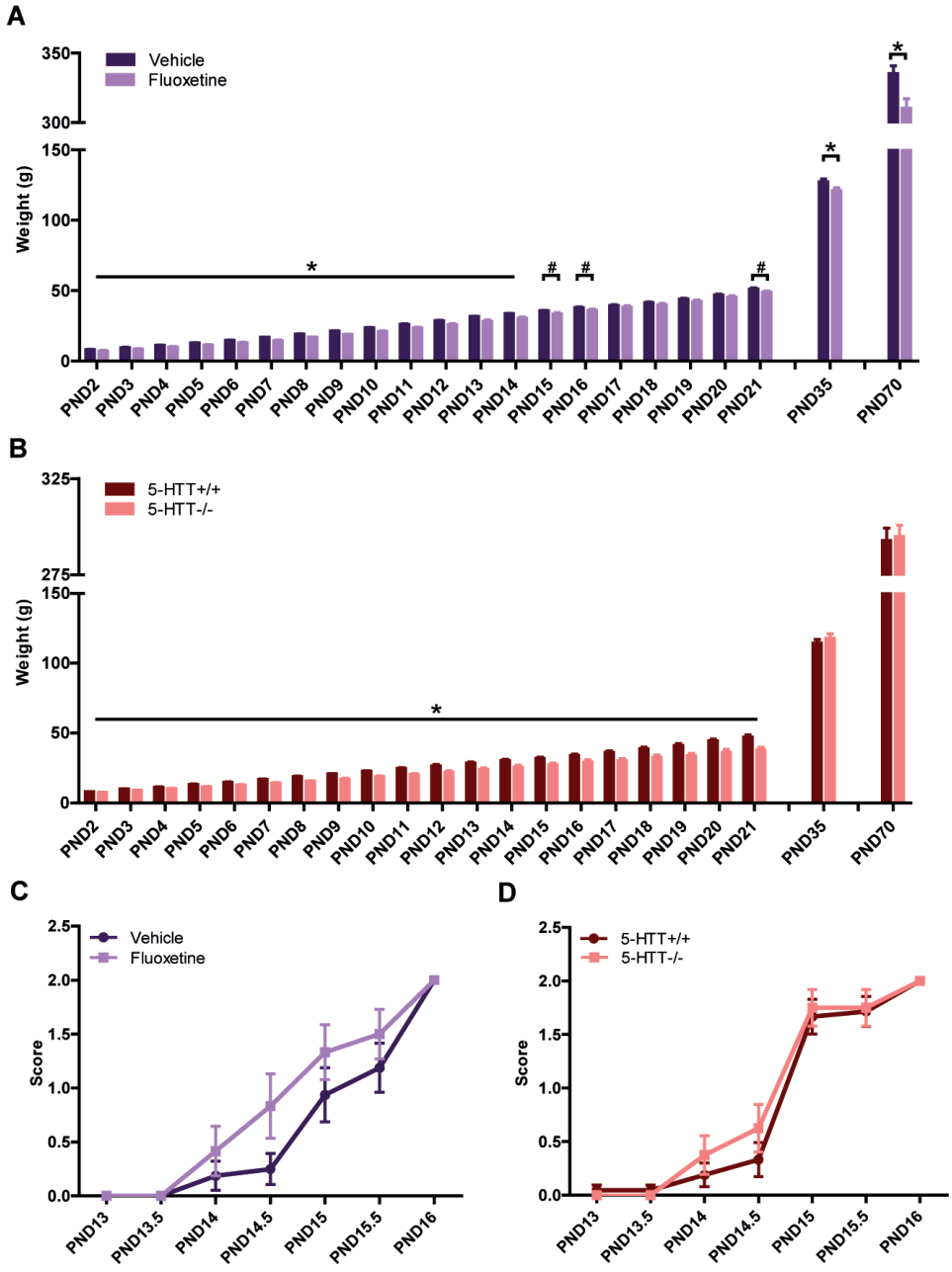

Figure 1. Effect of pharmacological and genetic inactivation of the 5-HTT on postnatal morphological development. Body weight in 5-HTT$^{+/+}$ (n=21/n=11) and 5-HTT$^{-/-}$ (n=16/n=12) male rats (A) and in perinatally fluoxetine- (n=12) and vehicle-exposed (n=16) male rats (B) measured in early-life (postnatal day (PND) 2 to 21), adolescence (PND35) and adulthood (PND70). Data are presented as mean + S.E.M. of body weight (g). Eye opening in 5-HTT$^{+/+}$ (n=21) and 5-HTT$^{-/-}$ (n=16) male rats (C) and in perinatally fluoxetine- (n=12) and vehicle-exposed (n=16) male rats (D) scored from PND13 to PND16 twice a day ((0) both eyes closed; (1) one eye open; (2) two eyes open). Data are presented as mean ± S.E.M. of score. *p<0.05, $^{\#}$p<0.1.

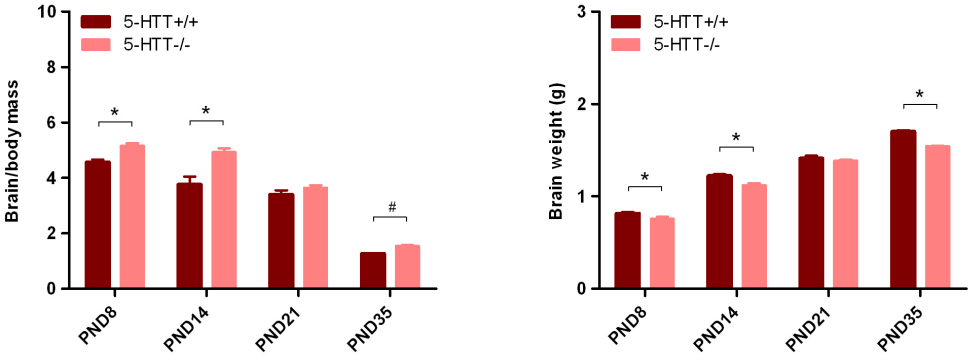

Figure 2. Effect of genetic inactivation of the 5-HTT on brain weight and brain/body mass index. Brain/body mass index (A) and brain weight (B) in 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ male rats measured at four time points. Brain/body mass index: PND8 (5-HTT$^{-/-}$ n=14, 5-HTT$^{+/+}$ n=16), PND14 (5-HTT$^{-/-}$ n=15, 5-HTT$^{+/+}$ n=15), PND21 (5-HTT$^{-/-}$ n=10, 5-HTT$^{+/+}$ n=6 and PND35 (5-HTT$^{-/-}$ n=2, 5-HTT$^{+/+}$ n=2). Brain weight: PND8 (5-HTT$^{-/-}$ n=14, 5-HTT$^{+/+}$ n=16), PND14 (5-HTT$^{-/-}$ n=15, 5-HTT$^{+/+}$ n=14), PND21 (5-HTT$^{-/-}$ n=10, 5-HTT$^{+/+}$ n=6 and PND35 (5-HTT$^{-/-}$ n=3, 5-HTT$^{+/+}$ n=4). Data are presented as mean + S.E.M. of weight (g). *p<0.05, #p<0.1.

p<0.1). Independent samples *t* tests showed that 5-HTT$^{-/-}$ rats turned significantly slower on PND8 ($t_{(1,18.81)}$=3.55; p<0.05) and showed a trend for turning slower on PND4, 7, 9 and 10 (p<0.1) (Figure 3F). Startle reflex was assessed on PND21, 35 and 69. The perinatally fluoxetine-exposed rats showed a significant decrease in startle reflex compared to the vehicle-exposed rats on PND21 (*U*=52.00, p<0.05), a trend for an increase on PND35 (*U*= 58.00, p<0.1) and a significant increase on PND69 (*U*=53.00, p<0.05) (Figure 3G). 5-HTT$^{-/-}$ rats (group 2) showed a significantly decreased startle reflex compared to 5-HTT$^{+/+}$ rats on PND21 (*U*=70.00, p<0.05), while no significant difference was found on PND35 and 69 (Figure 3H). In conclusion, both 5-HTT$^{-/-}$ and perinatally fluoxetine-exposed rats showed a delay in reflex development compared to control rats.

Effect of genetic and pharmacological 5-HTT inactivation on muscular strength and motor coordination

Motor coordination was tested by scoring swimming ability daily between PND8 and PND14. Perinatally fluoxetine-exposed rats displayed an impaired swimming development compared to vehicle exposed rats on PND8, PND9 and PND14 (p<0.05) and a trend on PND13 (p<0.1) (Figure 4A). 5-HTT$^{-/-}$ rats (group 1) showed impaired swimming ability compared to 5-HTT$^{+/+}$ rats on all days (p<0.05), as revealed by Fisher's exact test (Figure 4B). Swimming videos were re-analyzed by a second researcher resulting in similar results (data not shown). Muscle strength was analyzed from PND10 to PND19 and on PND21 using a bar holding test. Repeated measures ANOVA in rats perinatally exposed to fluoxetine or vehicle showed a significant effect on age ($F_{(4.90,107.71)}$=7.44; p<0.05), on treatment ($F_{(1,22)}$=9.57; p<0.05) and a significant age x treatment interaction ($F_{(4.90,107.71)}$=3.09; p<0.05). Independent samples *t* tests indicated

that the perinatally fluoxetine-exposed rats were able to hold the 3 mm bar significantly shorter ($p<0.05$) compared to the vehicle-exposed rats from PND10 to PND15 (Figure 4C). In the 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats (group 2), repeated measures ANOVA showed a significant age effect ($F_{(6.24,205.88)}=25.34$; $p<0.05$), genotype effect ($F_{(1,33)}=82.68$; $p<0.05$) and age x genotype interaction ($F_{(6.24,205.88)}=9.72$; $p<0.05$). Independent samples *t* tests demonstrated that 5-HTT$^{-/-}$ rats were able to hold the 3 mm bar significantly shorter compared to the 5-HTT$^{+/+}$ rats on all test days ($p<0.05$) (Figure 4D). The 5 mm bar showed similar results (Supplementary Figure 3). Furthermore, tracking the rats for 14 minutes (in the ODB and NOR tests) showed significantly reduced locomotion (distance moved) in the perinatally fluoxetine-exposed rats compared to the vehicle-exposed rats on PND34 ($t_{(1,26)}=2.16$; $p<0.05$). No significant treatment effect was found for locomotion in early-life (PND20) and adulthood (PND69) in this group (Figure 4E). The 5-HTT$^{-/-}$ rats (group 2 and 3) showed significantly reduced locomotion compared to the 5-HTT$^{+/+}$ rats on PND20 ($t_{(1,31)}=2.04$; $p<0.05$) and PND69 ($t_{(1,21)}=2.25$; $p<0.05$) and there was a trend for reduced locomotion in the 5-HTT$^{-/-}$ rats on PND34 ($t_{(1,21)}=2.00$; $p<0.1$) (Figure 4F). In addition, we measured unstable walking from PND3 to PND14. The perinatally fluoxetine-exposed rats showed no significant unstable walking after correcting for multiple testing (Figure 4G). The 5-HTT$^{-/-}$ rats (group 1) showed significantly more unstable walking compared to the 5-HTT$^{+/+}$ rats from PND3 to PND9 and on PND13 ($p<0.05$), indicating that unstable walking was more visible in the 5-HTT$^{-/-}$ group (Figure 4H). Videos of the walking test were re-analyzed by a second researcher resulting in similar results (data not shown). Taken together, both 5-HTT$^{-/-}$ and perinatally fluoxetine-exposed rats displayed defects in muscle strength and motor-related behavior.

Effect of genetic and pharmacological 5-HTT inactivation on olfactory discrimination

Olfactory function was tested on PND8, 10 and 12 using the olfactory discrimination task. In the rats perinatally exposed to fluoxetine or vehicle repeated measures ANOVA revealed a significant age effect ($F_{(1.15,24.06)}=10.48$; $p<0.05$), treatment effect ($F_{(1,21)}=88.05$; $p<0.05$) and age x treatment interaction ($F_{(1.15,24.06)}=4.62$; $p<0.05$). Independent samples *t* tests showed a trend for an increased latency to reach the bedding from their homecage for the fluoxetine-exposed rats compared to the vehicle-exposed rats on PND8 ($t_{(1,25)}= 2.32$; $p<0.1$) and on PND10 ($t_{(1,25)}= 1.99$; $p<0.1$) (Figure 5A). In the 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats (group 1) repeated measures ANOVA demonstrated a significant age effect ($F_{(1.74,60.77)}=10.296$; $p<0.05$), genotype effect ($F_{(1,35)}=26.495$; $p<0.05$) and age x genotype interaction ($F_{(1.74,60.77)}=10.675$; $p<0.05$). Independent samples *t* tests indicated that the latency to reach the bedding from their homecage is significantly increased in 5-HTT$^{-/-}$ rats compared to the 5-HTT$^{+/+}$ rats on PND8 ($t_{(1,35)}= 4.57$; $p<0.05$) and on PND10 ($t_{(1,35)}= 2.43$; $p<0.05$) (Figure 5B). Besides an increased latency to reach the bedding from their homecage, there were also 5-HTT$^{-/-}$ rats who walked to the fresh sawdust instead of to their homecage bedding on PND8 (n=3) and PND10 (n=3), while none of the 5-HTT$^{+/+}$ rats showed this behavior. In conclusion, these data showed that

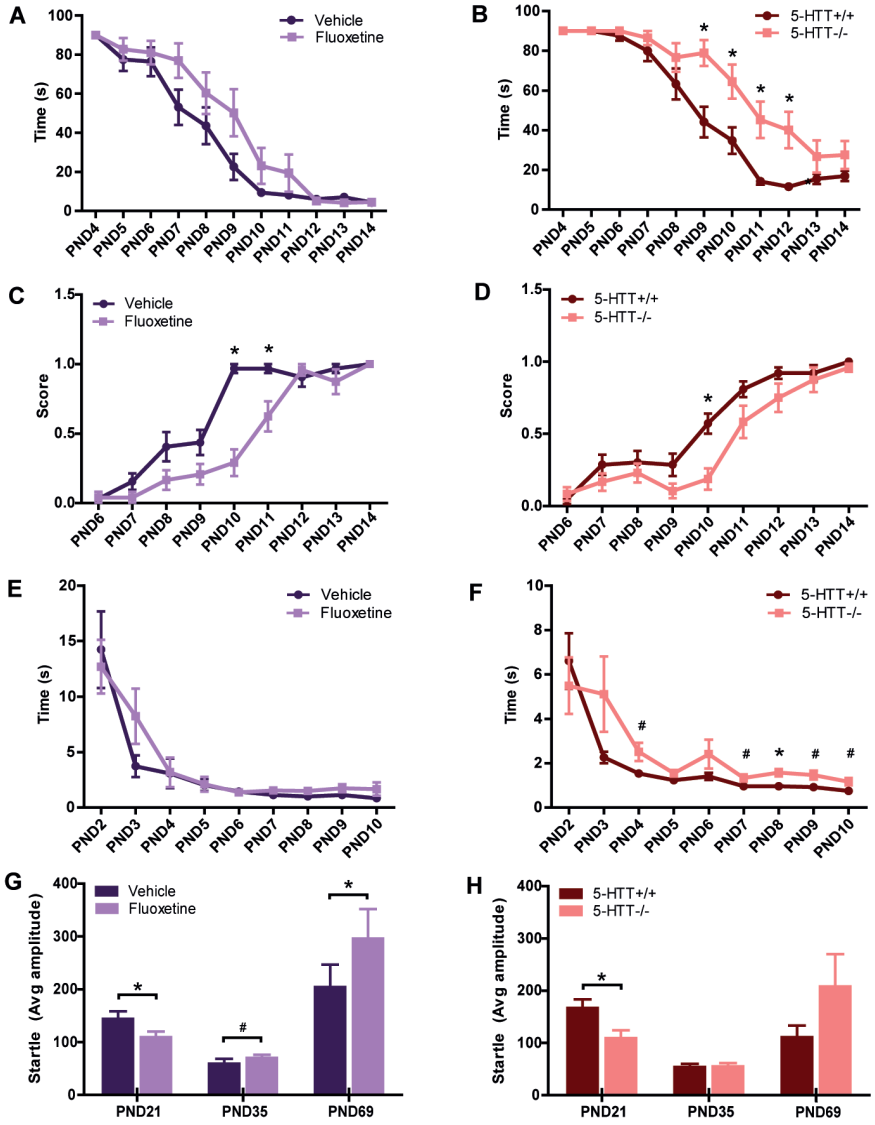

Figure 3. Effect of pharmacological and genetic inactivation of the 5-HTT on reflex development. Negative geotaxis in 5-HTT$^{+/+}$ (n=21) and 5-HTT$^{-/-}$ (n=16) male rats (A) and in perinatally fluoxetine- (n=12) and vehicle-exposed (n=16) male rats (B) was measured from postnatal day (PND) 4 to 14. Data are presented as mean ± S.E.M. of time turning 180° (s). Vibrissa placement in 5-HTT$^{+/+}$ (n=21) and 5-HTT$^{-/-}$ (n=16) male rats (C) and in perinatally fluoxetine- (n=12) and vehicle-exposed (n=16) male rats (D) was scored from PND6 to 14 ((0) no reflex; (1) reflex). Data are presented as mean ± S.E.M. of score. Righting reflex in 5-HTT$^{+/+}$ (n=21) and 5-HTT$^{-/-}$ (n=16) male rats (E) and in perinatally fluoxetine- (n=12) and vehicle-exposed (n=16) male rats (F) was measured from PND2 to 10. Data are presented as mean ± S.E.M. of righting time (s). Startle reflex in 5-HTT$^{+/+}$ (n=19/n=11) and 5-HTT$^{-/-}$ (n=17/n=11) male rats (G) and in perinatally fluoxetine- (n=12) and vehicle-exposed (n=16) male rats (H) was measured on PND21, 35 and 69. Data are presented as mean + S.E.M. of Avg startle amplitude. *p<0.05, $^{\#}$p<0.1.

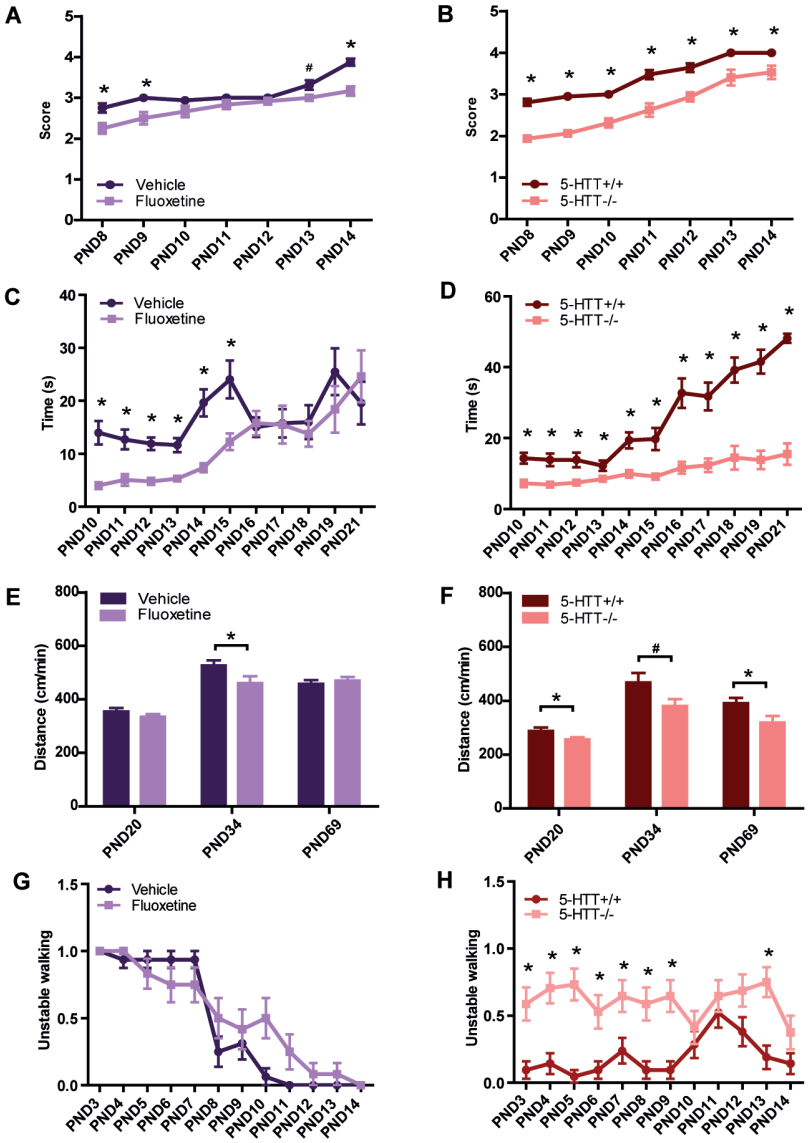

Figure 4. Effect of pharmacological and genetic inactivation of the 5-HTT on motor function and muscle strength. Swimming development in 5-HTT$^{+/+}$ (n=21) and 5-HTT$^{-/-}$ (n=16) male rats (A) and in perinatally fluoxetine- (n=12) and vehicle-exposed (n=16) male rats (B) was measured on postnatal day (PND) 8 to 14 (scoring: (1) head is fully under water; (2) back of the head is above water's surface, but ears are still partially under water and nose is pointing downwards; (3) nose above the water's surface, but ears still partially under water or (4) entire head remains above water's surface). Data are presented as mean ± S.E.M. of score. Bar holding on a 3 mm bar in 5-HTT$^{+/+}$ (n=19) and 5-HTT$^{-/-}$ (n=17) male rats (C) and in perinatally fluoxetine- (n=12) and vehicle-exposed (n=16) male rats (D) was measured on PND10-19, 21. Data are presented as mean ± S.E.M. of time holding the bar (s). Distance moved in 5-HTT$^{+/+}$ (n=19/n=11) and 5-HTT$^{-/-}$ (n=17/n=12) (E) and in perinatally fluoxetine- (n=12) and vehicle-exposed (n=16) male rats (F) was measured on PND20, 34 and 69. Data are presented as mean + S.E.M. of distance moved per minute (cm/min). Unstable walking in 5-HTT$^{+/+}$ (n=21) and 5-HTT$^{-/-}$ (n=16) male rats (G) and in perinatally fluoxetine- (n=12) and vehicle-exposed (n=16) male rats (H) was measured from PND3 to PND14. Scoring was as follows: (1) Shaking and unstable walking present, (0) no unstable walking and shaking. Data are presented as mean ± S.E.M. of walking score. *p<0.05, $^{\#}$p<0.1

the development of olfactory function is delayed in 5-HTT$^{-/-}$ rats and a similar pattern is seen in perinatally fluoxetine-exposed rats.

Effect of genetic and pharmacological 5-HTT inactivation on grooming behavior

Grooming behavior was assessed by scoring the total duration, latency and frequency on PND14,17,21, 35 and 69. In the rats perinatally exposed to fluoxetine or vehicle, repeated measures ANOVA revealed an age effect ($F_{(1.76,45.81)}$=37.31; p<0.05) in early-life (PND14,17 and 21), but no treatment effect or interaction was found. In adolescence (PND35) there was no significant difference, but in adulthood (PND69) the perinatally fluoxetine-exposed rats showed a significantly reduced grooming time ($t_{(1,25.49)}$=2.37; p<0.05) (Figure 5C). No differences were found in frequency and latency of grooming in this group (Supplementary Figure 4A/C). In the 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats (group 1) repeated measures ANOVA demonstrated an age effect ($F_{(1.48,50.16)}$=22.07; p<0.05) and a genotype effect $F_{(1,34)}$=37.63; p<0.05) in early-life (PND14,17 and 21). Independent samples *t* tests showed that the grooming time in 5-HTT$^{-/-}$ rats was significantly shorter on all test days (p<0.05). Also in adolescence (PND35) the grooming time in 5-HTT$^{-/-}$ rats (group 3) was significantly shorter ($t_{(1,21)}$=2.34; p<0.05), while there was no significant difference in adulthood (PND69) (Figure 5D). Likewise, there was a significant genotype effect ($F_{(1,34)}$=8.23; p<0.05) and age x genotype interaction ($F_{(2,68)}$=6.82; p<0.05) for the grooming latency. Independent samples *t* tests showed that the grooming latency in 5-HTT$^{-/-}$ rats was significantly increased on PND14 ($t_{(1,22.70)}$=2.47; p<0.05) and a trend for an increase was seen on PND17 ($t_{(1,35)}$=2.17; p<0.1). No differences in latency were seen in adolescence and adulthood (Supplementary Figure 4B). Frequency of grooming was also significantly reduced in the 5-HTT$^{-/-}$ rats compared to the 5-HTT$^{+/+}$ rats on PND14 (p<0.05) and 17 (p<0.05) and a trend for a reduction was seen on PND35 (p<0.1) using a Fisher's exact test (Supplementary Figure 4D). In conclusion, grooming behavior was mainly affected in 5-HTT$^{-/-}$ rats.

Effect of genetic and pharmacological 5-HTT inactivation on object directed behavior and novel object recognition

Restricted/repetitive interests[80] and cognition were assessed using the ODB and NOR test, respectively. No significant differences in ODB were found in early-life, adolescence and adulthood in rats perinatally exposed to fluoxetine or vehicle using independent sample *t* tests (Figure 5E). The 5-HTT$^{-/-}$ rats (group 3) showed significantly increased object directed behavior on PND34 ($t_{(1,15.15)}$=3.24; p<0.05) and PND69 ($t_{(1,17)}$=2.61; p<0.05) compared to 5-HTT$^{+/+}$ rats (group 3), while no difference was found on PND20 (group 2) (Figure 5F). For the NOR test no differences were found in rats perinatally exposed to fluoxetine or vehicle using independent samples *t* test (Figure 5G). The ability to discriminate old and new objects (as measured by the d2 index) was decreased in the 5-HTT$^{-/-}$ rats compared to 5-HTT$^{+/+}$ rats (group 3) on PND34 ($t_{(1,21)}$=2.21; p<0.05) and PND69 ($t_{(1,19)}$=2.20; p<0.05). This test was unfortunately not performed on PND20 (group 2) (Figure 5H). Taken together, rats lacking the serotonin

transporter showed object directed behavior and impaired object recognition in adolescence and adulthood.

Effect of genetic and pharmacological 5-HTT inactivation on prepulse inhibition of the acoustic startle reflex in adolescence

Sensorimotor gating was assessed using PPI. In the rats perinatally exposed to fluoxetine or vehicle repeated measures ANOVA showed a significant effect of prepulse strength (dB) in early-life (PND21) and adulthood (PND69). The rats showed significantly more inhibition after a stronger prepulse at PND21 ($F_{(2,52)}$=20.98; p<0.05) and PND69 ($F_{(2,52)}$=17.20; p<0.05). No main effect of treatment or interaction was found. In adolescence (PND35) a trend for a significant prepulse x treatment interaction was found ($F_{(2,52)}$=2.74; p<0.1). The fluoxetine-exposed rats showed a constant percentage of inhibition over all prepulse strengths, while an increase in inhibition was seen in the vehicle-exposed rats after a stronger prepulse. Independent samples *t* tests for each prepulse strength did not reveal a significant difference in PPI between the perinatally fluoxetine and vehicle-exposed rats after correcting for multiple testing. No main effect of treatment or prepulse strength was found in adolescence (Figure 6A,C,E). In the 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats repeated measures ANOVA revealed a significant effect of prepulse strength (dB) in early-life (group 2), adolescence and adulthood (group 3). The rats showed significantly more inhibition after a stronger prepulse (PND21: $F_{(2,68)}$=67.32; p<0.05, PND34: $F_{(2,40)}$=82.29; p<0.05, PND69: $F_{(1.45, 30.41)}$=44.15; p<0.05). Furthermore, in adolescence (PND34) a significant genotype effect ($F_{(1,20)}$=6.46; p<0.05) and genotype x prepulse strength interaction ($F_{(2,40)}$=4.97; p<0.05) was found. Independent samples *t* tests demonstrated that prepulse inhibition was stronger in 5-HTT$^{-/-}$ rats compared to 5-HTT$^{+/+}$ rats for a prepulse of 5 dB ($t_{(1,11.88)}$=2.72; p<0.05) and 10 dB ($t_{(1,20)}$=-2.83; p<0.05) above background noise (Figure 6B,D,F). In conclusion, only in adolescence PPI is affected in 5-HTT$^{-/-}$ and perinatally fluoxetine-exposed rats.

Analysis of correlations between aberrant behaviors

To investigate whether there is a correlation between the behaviors we measured, we performed a correlation analysis. Since numerous correlations could be performed, we decided to test only correlations between behavioral tests performed at the same day or if not possible with a maximum of one day difference. Overall, the correlations of behaviors in different tests in early-life were stronger in the 5-HTT knockout group compared to the fluoxetine group. We will only discuss part of the correlations here, for a complete list of correlation values see Online Supplementary Table 1. In rats perinatally exposed to fluoxetine or vehicle, body weight was positively correlated with swimming on PND9, 10, 11 and 13 and with bar holding on PND10 and a trend on PND12, 14 and 17. In the 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats body weight was also positively correlated with swimming behavior from PND8 to PND14 (p<0.05) and with bar holding on PND10 and PND13 to PND20 (p<0.05). A trend was seen with bar holding on PND11 and PND12 (p<0.1). Furthermore, in the 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats

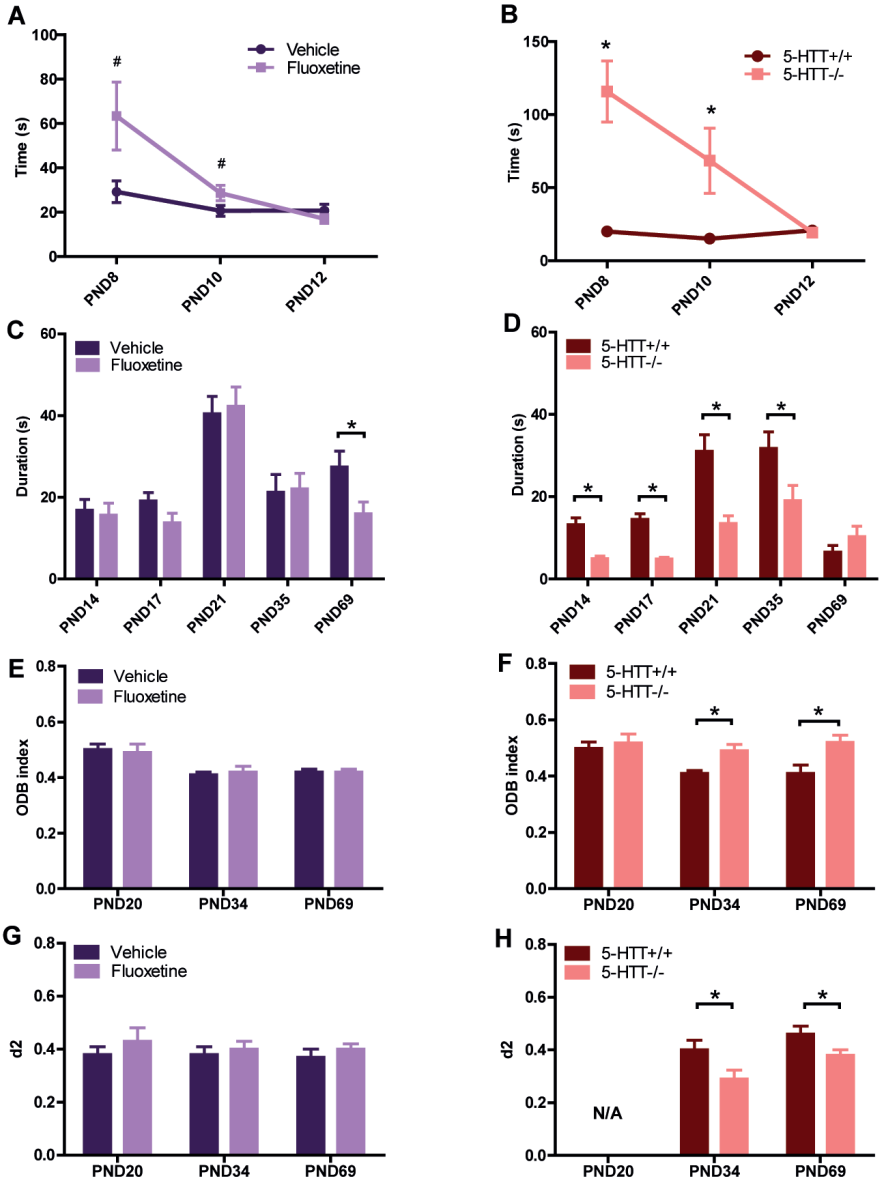

Figure 5. Effect of pharmacological and genetic inactivation of the 5-HTT on olfactory function, grooming, restricted and cognitive behavior. Olfactory discrimination in 5-HTT$^{+/+}$ (n=21) and 5-HTT$^{-/-}$ (n=16) male rats (A) and in perinatally fluoxetine- (n=12) and vehicle-exposed (n=16) male rats (B) was measured from postnatal day (PND) 8 to 12. Data are presented as mean ± S.E.M. of latency to reach familiar sawdust (s). Grooming time in 5-HTT$^{+/+}$ (n=21/n=11) and 5-HTT$^{-/-}$ (n=16/n=12) male rats (C) and in perinatally fluoxetine- (n=12) and vehicle-exposed (n=16) male rats (D) was measured on PND14, 17, 21, 35, 69. Data are presented as mean + S.E.M. of grooming duration (s). For the object directed behavior (ODB) test, time exploring each object was measured on PND20, 34 and 69 in 5-HTT$^{+/+}$ (n=19/n=11) and 5-HTT$^{-/-}$ (n=17/n=12) male rats (E) and in perinatally fluoxetine- (n=12) and vehicle-exposed (n=16) male rats (F) and ODB was calculated (exploration time most explored object/total exploration time). Data are presented as mean + S.E.M. of ODB. For the novel object recognition (NOR) test, time exploring each object was measured in 5-HTT$^{+/+}$ (n=11) and 5-HTT$^{-/-}$ (n=12) male rats (G) and in perinatally fluoxetine- (n=12) and vehicle-exposed (n=16) male rats (H) on PND20, 34 and 69 and NOR was calculated (exploration time new object/total exploration time). Data are presented as mean + S.E.M. of NOR. *p<0.05, $^{\#}$p<0.1

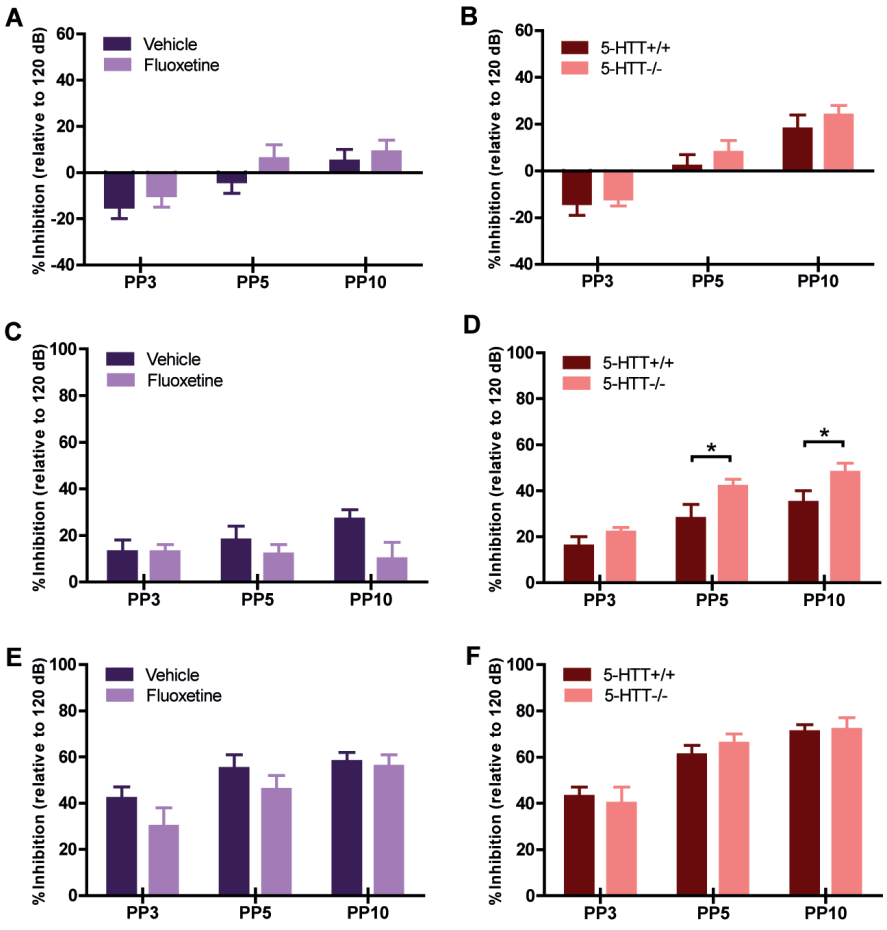

Figure 6. Effect of pharmacological and genetic inactivation of the 5-HTT on prepulse inhibition. Prepulse inhibition (PPI) of the acoustic startle reflex was measured in 5-HTT$^{+/+}$ (n=19/n=11) and 5-HTT$^{-/-}$ (n=17/n=12) male rats on postnatal day (PND)21 (A), PND35 (C) and PND69 (E) and in perinatally fluoxetine- (n=12) and vehicle-exposed (n=16) male rats on PND21 (B), PND35 (D) and PND69 (F). PPI was calculated according to the formula: 100 – (Average of all startle amplitude on prepulse trial/ Startle amplitude on startle trial) × 100. Three different prepulses were tested; 3, 5, or 10 dB above background noise. *p<0.05

body weight was also positively correlated with startle reflex on PND21 ($r_{(36)}$=0.391, p<0.05) and grooming time on PND17 ($r_{(35)}$=0.385, p<0.05) and PND21 ($r_{(37)}$=0.560, p<0.05). In addition, in the 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats bar holding on PND21 was positively correlated with startle reflex ($r_{(36)}$=0.41, p<0.05) on PND21 and locomotion on PND20 ($r_{(36)}$=0.33, p<0.05). We also observed that in the 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats swimming was positively correlated with grooming on PND14 ($r_{(37)}$=0.570, p<0.05) and with vibrissa placing on PND9 ($r_{(37)}$=0.36, p<0.05) and PND10 ($r_{(37)}$=0.33, p<0.05) and a trend on PND13 ($r_{(37)}$=0.31, p<0.1). A negative correlation was found between swimming and geotaxis on PND9 ($r_{(37)}$=-0.39, p<0.05),10 ($r_{(37)}$=-0.33, p<0.05) and 11 ($r_{(37)}$=-0.36, p<0.05). Later in life, we found in the 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats

a negative correlation between grooming time and ODB in adolescence ($r_{(23)}$=0.56, p<0.05) and a trend was seen in adulthood ($r_{(23)}$=0.35, p<0.1). This negative correlation between ODB and grooming time was also found in the fluoxetine group, but only in adulthood ($r_{(28)}$=-0.383, p<0.05).

Discussion

In this study, we demonstrate that both pharmacological (perinatal SSRI exposure) and genetic (knockout) 5-HTT inactivation result in a developmental delay. Specifically, we observed a delay in reflex development and olfactory function. In addition, we found defects in grooming and motor-related behavior and a decrease in body weight. Most of the observed early-life effects were normalized later in life. Interestingly, in adolescent and adult 5-HTT$^{-/-}$ rats, but not in fluoxetine-exposed rats, we observed object directed behavior and decreased novel object recognition.

We are the first to report the aberrant developmental time-course in early-life of 5-HTT$^{-/-}$ rodents. We observed a delay in reflex development, as shown in the righting reflex, negative geotaxis, vibrissa placing and startle reflex. In addition, we found muscle and motor-related changes in the 5-HTT$^{-/-}$ rats compared to the 5-HTT$^{+/+}$ rats, like hypolocomotion, unstable walking, decreased bar holding time and lower swimming scores. Furthermore, olfactory discrimination and grooming development were delayed in the 5-HTT$^{-/-}$ rats. We also found a reduction in body weight in the 5-HTT$^{-/-}$ rats during early life, which has been shown before[85]. All differences seen in the reflex tests were normalized before adolescence (PND35). Regarding motor-related changes, the 5-HTT$^{-/-}$ rats showed hypolocomotion also during adolescence and adulthood. Unfortunately, walking and swimming behavior were not tested after PND14 and our bar holding setup did not give reliable results in adolescence due to the fact that the rats could see the ground (eyes open) and jumped off the bar. While most of the early-life differences were normalized in adolescence, differences in PPI, ODB and NOR were only seen later in life (adolescence and adulthood). Why we only see these differences later in life is unclear, but it might be related to the refinement of dendritic branching and synaptic connections which is occurring around adolescence[86]. Serotonin is involved in the regulation of synaptogenesis and the elimination of exuberant undesired connections, known as synaptic pruning[10,87,88]. It has been shown that 5-HTT$^{-/-}$ mice display increased spine densities during adulthood, which can be a result from pruning deficits[89]. Pruning is affected by serotonin's capability to inhibit the activation of microglia via 5-HT2 receptor activation, resulting in reduced uptake of synaptic particles[90]. In addition, the 8 hour interval in the NOR test might be optimal for testing recognition memory later in life, but not for early-life (See also Reger et al.[91]). Increased object directed behavior in the 5-HTT$^{-/-}$ rats reflects repetitive/restricted interests which is a sign of autistic-like behavior[80]. Increased PPI and decreased NOR (shown before in our adult 5-HTT$^{-/-}$ rats[92]) are seen in animal models for autism spectrum disorder[93,94]. This observation underscores the postulated link between early-life increased extracellular serotonin levels and autism spectrum disorder[42-44]. Serotonin is shown to be

modulating several pathways associated with autism spectrum disorder, like the mTOR pathway[95-97], glutamate signaling[98,99] and neurotrophin signaling[100,101], which strengthens our autism-related behavior findings. An interesting example is the mTOR pathway. Hyperactivation of this pathway is associated with autism[96,97]. The serotonin receptor 5-HT6 can form a complex with mTORC1 and activate the mTOR pathway, suggesting that increased serotonin signaling can also hyperactivate the mTOR pathway[95]. Furthermore, it has been shown that a hyperactivated mTOR pathway can cause pruning defects[102] and both increased extracellular serotonin and autism spectrum disorder are associated with increased dendritic spine density caused by reduced developmental spine pruning[102,103]. However, there are also studies showing decreased PPI and increased grooming in autism spectrum disorder[93,104-107], which are not seen in our 5-HTT$^{-/-}$ rats. Besides the behavior, we observed an increase in brain/body mass index in the 5-HTT$^{-/-}$ rats compared to the 5-HTT$^{+/+}$ rats, which is also shown by Page and colleagues[108] in 5-HTT$^{+/-}$ mice and is linked to autism spectrum disorder. Hence, more (behavioral) tests are needed to confirm whether the 5-HTT$^{-/-}$ rats display phenotypes of autism spectrum disorders.

As mentioned in the introduction, there are several studies showing effects of perinatal SSRI exposure on neurodevelopmental behavior. While the results in human studies are often based on a combination of different SSRI types and exposure periods, animal studies have shown that the type of SSRI and exposure period might result in different behavioral outcomes[57,109,110]. We studied the effects of fluoxetine exposure from GD11 to PND7 on neurodevelopmental behavior. So far, studies using pre- and postnatal fluoxetine exposure reported a reduction in body weight[111] and sexual behavior[112,113] and an increase in anxiety[111,114] and depression-like behavior[111] in the offspring. We also observed the reduction in body weight in the fluoxetine-exposed rats from early-life till adulthood (the reduction was not significant from PND17 to PND21 probably because of increased variation in body weight). Furthermore, we found a delay in reflex development in the fluoxetine-exposed rats compared to the vehicle-exposed rats, as reflected by an increased turning latency in the negative geotaxis test, reduced vibrissa placing and decreased startle reflex. This delay corresponds well with findings of Deiro and colleagues, who found a delay in reflex development in rats postnatally (PND1-PND21) exposed to sertraline or citalopram[58,59]. Increased turning latencies in negative geotaxis were also shown by Zimmerberg and Germeyan[61] in prenatally fluoxetine-exposed rats. In addition, the fluoxetine-exposed rats showed reduced performance in the bar holding test and swimming test in early-life, and they showed hypolocomotion in adolescence. Changes in motor-related behavior (bar holding, hypolocomotion) were observed before in rats postnatally exposed to fluoxetine[60,62], indicating that 5-HT levels in the postnatal period are important for development of motor functions. As in the 5-HTT$^{-/-}$ rats, most of the early-life differences seen in the fluoxetine-exposed rats where normalized at adolescent stage. In adolescence we found a trend for a treatment x prepulse interaction in PPI. In adulthood we found an increased startle reflex in the fluoxetine-exposed rats which might reflect anxiety-like behavior[115].

We observed similar effects on reflex development and muscle/motor-related behavior in the perinatally fluoxetine-exposed rats and 5-HTT$^{-/-}$ rats. Overall, the effects on olfactory function, grooming and muscle and motor-related behavior were stronger in the 5-HTT$^{-/-}$ rats compared to the fluoxetine-exposed rats. Differences between the two models might be caused by the fact that 5-HTT is only transiently blocked by fluoxetine, while it is lifelong absent in the 5-HTT$^{-/-}$ rats. In addition, fluoxetine has weak affinity for the norepinephrine and dopamine transporters[116,117], which might also contribute to the effects on early-life behavior. Differences between the models are seen in adolescence and adulthood, which might be related to increased 5-HT levels in the 5-HTT$^{-/-}$ rats over their entire life span, whereas in the perinatally fluoxetine-exposed rats elevated levels are restricted to the perinatal period. Object directed behavior and a lower d2 in NOR were only seen in the 5-HTT$^{-/-}$ versus 5-HTT$^{+/+}$ rats (in adolescence and adulthood). PPI in adolescence was affected in both 5-HTT$^{-/-}$ versus 5-HTT$^{+/+}$ and fluoxetine- versus vehicle-exposed rats, but in opposite direction. 5-HTT$^{-/-}$ rats showed a stronger increase in percentage inhibition by increasing prepulse strength compared to 5-HTT$^{+/+}$ rats, while fluoxetine-exposed rats showed a trend towards a reduced increase in percentage inhibition by increasing prepulse strength compared to vehicle-exposed rats. The weak affinity of fluoxetine for the dopamine transporter might contribute to the decrease in PPI in the fluoxetine-exposed rats, as increased dopaminergic signaling can contribute to a decrease in PPI[118,119].

Rodents with impaired (early-life) 5-HTT function are often used as a model for depression and anxiety, however this depressive and anxious phenotype is mainly based on adult findings. As the focus of our study was on investigating neurodevelopmental behavior, we did not test anxiety and depression-like behavior across ages but it might very well be that the anxiety and depressive symptoms are only present later in life, while other symptoms are only present during early-life. We observed differences in motor-related behavior in early-life, suggesting that early-life reduction in 5-HTT function might be a good model for motor disorders in early-life. Since the serotonergic system is involved in energy regulation[120], it might be that rodents with impaired (early-life) 5-HTT function use their energy for different processes in early-life compared to adulthood. The development of motor function can be delayed, while other (unknown) functions are well- (or over) developed.

We should note that additional factors might contribute to the results of some of the behavioral tests, complicating the interpretation of the findings. Especially differences in motor function can affect several behavior tests, like righting reflex, negative geotaxis, olfactory discrimination (but we do see that some of the 5-HTT$^{-/-}$ rats walk to the fresh sawdust) and startle reflex. We showed that there is a positive correlation between bar holding and startle reflex in the 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats, indicating that the jumping in the startle test might be influenced by muscle strength. In addition, in the 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats there was a significant negative correlation between swimming and negative geotaxis, suggesting that increased turning latency in the negative geotaxis test might be caused by defects in motor function. We also found a positive correlation between grooming and swimming

suggesting that grooming behavior is dependent on motor function. In the fluoxetine group no strong correlations between motor or muscle tests and other behavioral tests were found. Furthermore, correlational analysis showed that body weight is positively correlated with several behavioral test, like bar holding, swimming, grooming and startle reflex. A worse performance with a lower body weight in bar holding, swimming, grooming and startle reflex might reflect a delay in development of muscles causing the lower body weight.

The strength of our study is that we performed a battery of behavior experiments under well controlled environments (temperature, noise level, type of SSRI, exposure period and dose) making it possible to monitor behavior across developmental stages. We used both perinatally SSRI-exposed and 5-HTT$^{-/-}$ rats to study different types of behavior, which enables direct examination of similarities and differences between these two models. However, there are also several limitations of the study. We performed multiple behavioral tests in the same group of rats. Repeated testing might influence the outcome of the tests (carry-over effect), although we do think that most of the behavioral tests are very mild and the sequence of the behavior tests and the interval between the tests was always the same in the test and control group. Furthermore, we did not test motor-related behavior in adolescence and adulthood (except locomotion). Testing rotarod or bar holding under dimmed or red light conditions might be appropriate ways to study motor-related behavior later in life. In addition, we observed contradictory results regarding differences in brain weight. We found an increase in brain/body mass index, but a reduction in brain weight *per se*. While the brain/body mass index is a commonly used measure, there are also studies looking at brain weight *per se* since body weight is a factor that can be influenced by various parameters (e.g. fat and muscles). It is important to mention that a lower brain weight *per se*, as seen in the 5-HTT$^{-/-}$ rats, is linked to major depressive disorder[121] and the serotonin system[122,123]. For mothers using SSRIs during pregnancy it is important to know whether SSRIs can cause livelong effects on neurodevelopmental behavior. In addition, testing neurodevelopmental behavior in the 5-HTT$^{+/-}$ rat, which is argued to best model the human 5-HTTLPR S-allele, might provide valuable additional information. Lastly, it would be of interest to investigate whether prenatal stress increases the differences in early-life behavior between the 5-HTT inactivation models and their controls, as is seen for depression-like symptoms after early-life stress in relation to the 5-HTTLPR s/s (and s/l) genotype[124-126].

In conclusion, we demonstrate that perinatal reduction of functional 5-HTT, by blocking 5-HTT using an SSRI or by genetically knocking out 5-HTT, results in a developmental delay. Strongest effects are observed in reflex development and motor-related behavior. Most of the early-life changes are normalized later in life, except for hypolocomotion. In adolescent and adult 5-HTT$^{-/-}$ rats we observe object directed behavior and decreased novel object recognition, which are not seen in the perinatally fluoxetine-exposed rats and might be related to the lifelong absence of 5-HTT. Together, these data provide important new insights into the effects of perinatal and lifelong 5-HTT inactivation on behavior across developmental stages.

Conflict of interest

The authors declare no conflict of interest.

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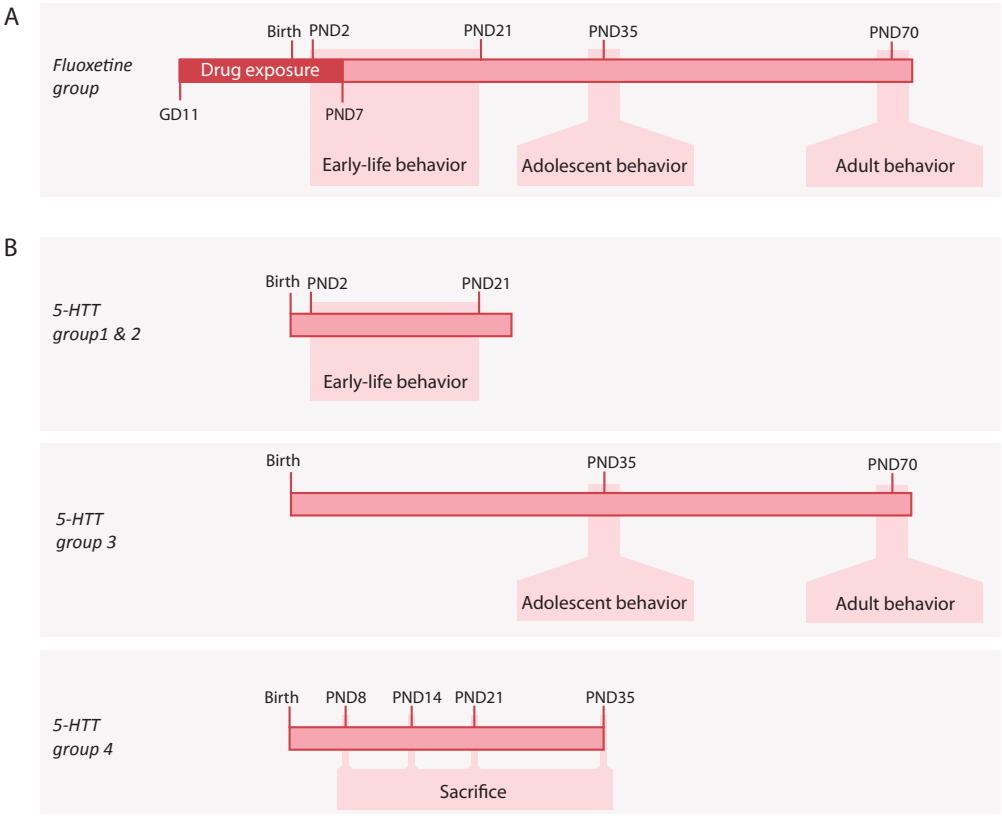
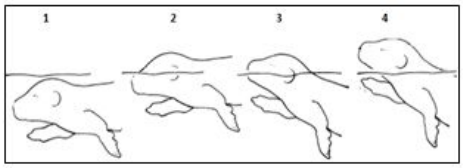
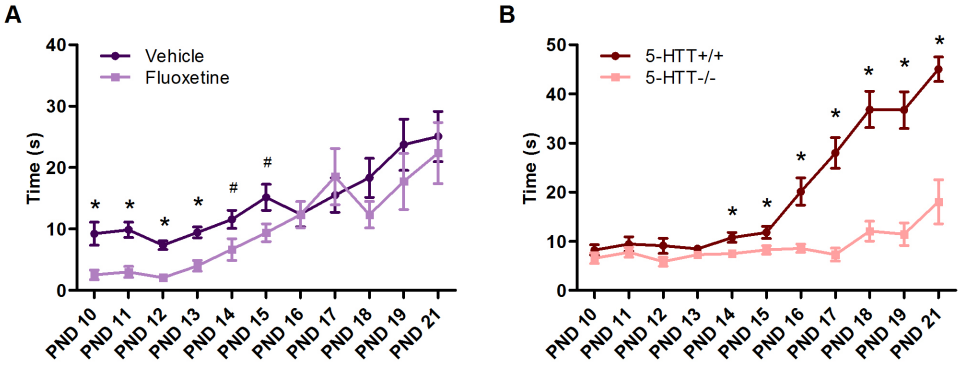
Supplementary material



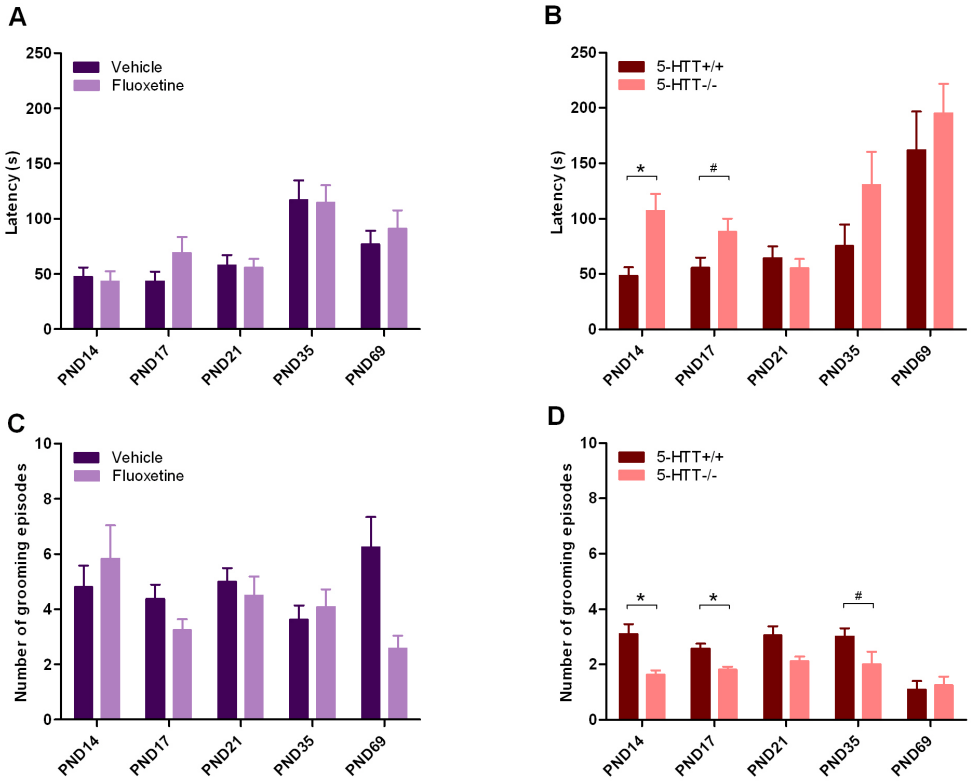
Supplementary Figure 1. Schematic representation of experimental timeline. Experiments performed in perinatally fluoxetine- and vehicle-exposed rats (A) and in 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ rats (B). For perinatal fluoxetine exposure, dams were treated from gestational day (GD) 11 to 7 days after delivery. Male offspring was used for the following behavior tests: body weight, eye opening, righting reflex, swimming, negative geotaxis, vibrissa placing, grooming, bar holding, object directed behavior (ODB), novel object recognition (NOR), startle reflex and prepulse inhibition (PPI). At postnatal day (PND) 22, pups were weaned and group-housed for further examination (2 rats/cage). Male Wistar rats in group 1 (5-HTT$^{+/+}$ n=21; 5-HTT$^{-/-}$ n=16) and 2 (5-HTT$^{+/+}$ n=19; 5-HTT$^{-/-}$ n=17) were used to test early-life behavior from PND2 to PND21. Rats in group 1 were used for body weight, eye opening, righting reflex, swimming, negative geotaxis, vibrissa placing and grooming. Male Wistar rats in group 2 were used for body weight, bar holding, ODB, NOR, startle reflex and PPI. Male Wistar rats in group 3 (5-HTT$^{+/+}$ n=11; 5-HTT$^{-/-}$ n=12) were used for testing adolescent (around PND35) and adult (around PND70) behavior. The following tests were performed: body weight, grooming, ODB, NOR, startle reflex and PPI. Male Wistar rats in group 4 were sacrificed at four different time points (PND8, 14, 21 and 35) and used for measuring brain weight.



Supplementary Figure 2. Scoring of swimming development. Swimming development was scored according to the following scores: (1) head is fully under water; (2) back of the head is above water's surface, but ears are still partially under water and nose is pointing downwards; (3) nose above the water's surface, but ears still partially under water or (4) entire head remains above water's surface. Scoring system is adapted from Schapiro and colleagues[1].



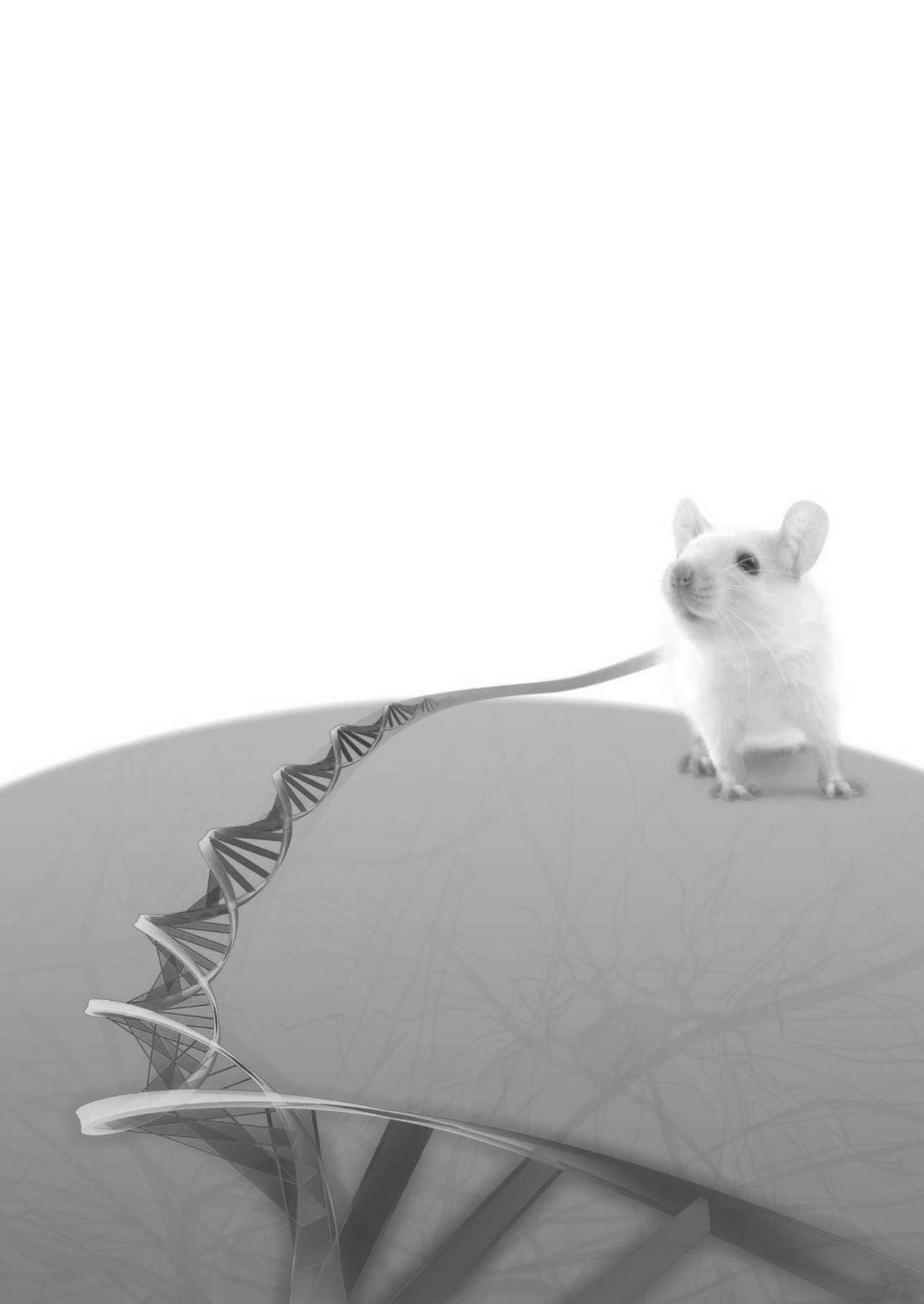
Supplementary Figure 3. Bar holding (5mm). Bar holding on a 5 mm bar in 5-HTT$^{+/+}$ (n=19) and 5-HTT$^{-/-}$ (n=17) male rats (A) and in perinatally fluoxetine- (n=12) and vehicle-exposed (n=16) male rats (B) was measured from PND10 to 19 and 21. Data are presented as mean ± S.E.M. of time holding the bar (s). *p<0.05, #p<0.1



Supplementary Figure 4. Grooming latency and frequency. Grooming latency (s) and number of grooming episodes in 5-HTT$^{+/+}$ (n=21/n=11) and 5-HTT$^{-/-}$ (n=16/n=12) male rats (A,C) and in perinatally fluoxetine- (n=12) and vehicle-exposed (n=16) male rats (B, D) was measured on PND14, 17, 21, 35, 69. Data are presented as mean + S.E.M. *p<0.05, $^{\#}$p<0.1

Supplementary references

1. Schapiro, S., Salas, M. & Vukovich, K. Hormonal effects on ontogeny of swimming ability in the rat: assessment of central nervous system development. *Science* **168**, 147-50 (1970).



5

Transcriptome analysis identifies multifaceted regulatory mechanisms dictating a genetic switch from neuronal network establishment to maintenance during postnatal prefrontal cortex development

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Abstract

The prefrontal cortex (PFC) is one of the latest brain regions to mature, which allows the acquisition of complex cognitive abilities through experience. To unravel the underlying gene expression changes during postnatal development we performed RNA-sequencing (RNA-seq) in the rat medial PFC (mPFC) at five developmental time points from infancy to adulthood, and analyzed the differential expression of protein-coding genes, long intergenic non-coding RNAs (lincRNAs) and alternative exons. We showed that most expression changes occur in infancy, and that the number of differentially expressed genes reduces toward adulthood. We observed 137 differentially expressed lincRNAs and 796 genes showing alternative exon usage during postnatal development. Importantly, we detected a genetic switch from neuronal network establishment in infancy to maintenance of neural networks in adulthood based on gene expression dynamics, involving changes in protein-coding and lincRNA gene expression as well as alternative exon usage. Our gene expression datasets provide insights into the multifaceted transcriptional regulation of the developing PFC. They can be used to study the basic developmental processes of the mPFC and to understand the mechanisms of neurodevelopmental and neuropsychiatric disorders. Our study provides an important contribution to the ongoing efforts to complete the 'brain map', and to the understanding of PFC development.

Introduction

Cortical development is a molecularly orchestrated process that begins prenatally and continues postnatally until late adolescence, arguably throughout the lifespan[1]. It consists of a complex series of dynamic processes that operate throughout the course of development to promote new neural structures and functions. The birth, proliferation, migration, neuronal cell death, differentiation and target selection (axonal guidance) of neurons occurs mainly prenatally[2-4]. Postnatal developmental processes consist of dendritic growth, dendritic and axonal branching, synaptogenesis, synaptic pruning and myelination[2,5-7]. Homeostatic control and metabolic regulation are the main processes taking place in the adult brain[8,9].

The prefrontal cortex (PFC) is one of the last regions of the brain to reach maturation[10]. The PFC receives input from many regions including all cortical regions and is involved in the execution of cognitive and emotional functions, such as decision-making, planning, social interaction and impulse control[11-15]. The prolonged development of the PFC allows the acquisition of complex cognitive abilities through experience[11].

The development of the PFC has often been studied in rodent models, because their development is faster than in humans, and it is easier to control environmental factors which can influence neurodevelopment. It has been shown that the sequence of key events in brain development is largely conserved between humans and rodents[16,17], which makes rodents useful models for studying neurodevelopmental processes. However, it should be noted that rats are born more immature than humans in terms of brain maturation[18]. The first postnatal week in rats roughly corresponds to the third trimester of human gestation[19]. In addition, studies comparing rodent and primate PFC show that cortical areas are not only less segregated in rats compared to primates[20], they are also less numerous overall[21,22]. Furthermore, some studies suggest that the relative size of the PFC is larger in humans than in other species[10,23-25]. These differences should be kept in mind when comparing rodent and human mPFC development.

While postnatal morphological changes in the PFC are well studied, less is known about the molecular changes underlying this. Several studies focused on the expression changes of protein-coding genes related to neurotransmitter systems (serotonin, gamma-aminobutyric acid (GABA), glutamate) and to neurotrophin signaling pathways (*Trkb, Bdnf*), showing different expression patterns during PFC development[26-29]. In addition, alternative exon usage is a process that occurs extensively in the nervous system to regulate receptor localization (e.g. *Grin1, Grm1*), axon guidance (e.g. *Dscam*) and synapse formation (e.g. *Agrn, Nrxn1, Nrxn2, Nrxn3*)[30-32]. Furthermore, long intergenic non-coding RNAs (lincRNAs) have been shown to be involved in neurodevelopmental processes, such as neural stem cell maintenance and differentiation (e.g. HOTAIRM), neurogenesis (e.g. RMST), gliogenesis (e.g. SOX8OT) and neural plasticity (e.g. NEAT2)[33-36]. LincRNAs have been implicated in the regulation of the expression of protein-coding genes[37], especially of genes in their genomic vicinity[38]. While most studies have focused on individual genes, several recent studies investigated the expression of protein-coding genes in the PFC on a genome-wide level using

gene expression microarrays[39-43]. In addition, the Allen brain atlas provides PFC expression data from a large set of genes at different time points during mouse[44], non-human primate[45] and human development[46,47]. So far, only one study investigated genome-wide alternative splicing in the human PFC during development using RNA-seq[48] and there are no studies focusing on lincRNA changes during PFC development. It is likely that changes in expression of protein-coding genes and lincRNAs as well as in the alternative exon usage of protein-coding genes together regulate PFC development, and therefore it is important to investigate the combination of these gene regulation mechanisms during PFC development on a genome-wide scale and in a temporal manner.

In this study, we performed transcriptome analysis during medial PFC (mPFC) development in male Wistar rats. We used different developmental time points (infancy (PND8, PND14), (pre-) adolescence (PND21, PND35) and adulthood (PND70)) and studied genome-wide protein-coding gene expression, lincRNA expression and alternative exon usage during development using RNA-sequencing (RNA-seq). We identify a genetic switch during mPFC development from neuronal network establishment in infancy to maintenance of neuronal networks in adulthood, which is supported by protein-coding and lincRNA gene expression dynamics as well as alternative exon usage patterns. Our study additionally provides a rich resource for future studies in neurodevelopmental and neuropsychiatric disorders.

Materials and methods

Animals

Wistar rats (*Rattus norvegicus*) were bred, reared and housed in individually ventilated cages (40x35x23, Greenline, Tecniplast, West Chester, Pennsylvania, USA) in temperature-controlled rooms (21 °C ± 1 °C) under standard 12-h light/dark cycle (lights on at 7:00 A.M.) with food (Sniff, long cut pellet, Bio Services, Uden, The Netherlands) and water available *ad libitum*. Pups were weaned at PND22. Male rats were sacrificed by decapitation at five different time points, PND8, PND14, PND21, PND35 and PND70. Three different nests per time point were used. All efforts were made to minimize animal suffering and to reduce the number of rats used. We used Wistar rats for this study because this is a commonly used rat strain in the laboratory. All experiments were carried out according to the guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council 2003), the principles of laboratory animal care, as well as the Dutch law concerning animal welfare.

RNA extraction and double-stranded cDNA synthesis

Five rat brain samples per time point were used for RNA extraction. mPFC tissue was dissected from seven (PND8 and 14) or eight (PND21, 35 and 70) consecutive slices of 200 µm using a 2 mm punch needle. More information about the exact position of the punches is shown in Supplementary Figure 1. Total RNA was isolated with QIAzol (RNeasy lipid tissue kit; QIAGEN, Venlo, The Netherlands) according to the manufacturer's recommendations. From each sample 2.5 µg RNA was used for rRNA depletion using the Ribo-Zero rRNA Removal Kit

(Human/Mouse/Rat, Epicentre, Madison, Wisconsin, USA) according to the manufacturer's recommendations. RNA fragmentation reactions were performed using fragmentation buffer (5x; 200 mM Tris-Ac, 500 mM potassium-Ac, 150 mM Magnesium-Ac, pH 8.2) in a final concentration of 1x per reaction. Fragmentation reactions were incubated at 95°C for 4 min on a thermal cycler and placed on ice for 10 min. Fragmented rRNA-depleted RNA was purified using ethanol precipitation. First and second strand synthesis was performed as described by Kouwenhoven and colleagues[49].

Sequencing

DNA samples were prepared for sequencing by end repair of 5 ng total DNA as measured by Qubit dsDNA HS (Invitrogen). NEXTflex adaptors (Bioo Scientific, Austin, Texas, USA) were ligated to the DNA fragments, followed by post-ligation cleanup using Agencourt AMPure XP beads (Beckman Coulter, Woerden, The Netherlands), library amplification by PCR (10 cycles) and size selection (~300 bp) using Agencourt AMPure XP beads (Beckman Coulter). Quality control of DNA libraries prepared for sequencing was performed by qPCR and by running the products on a Bioanalyzer (Bio-Rad, Veenendaal, The Netherlands). Cluster generation and sequencing (50 bp, single end reads) was performed with the Illumina HiSeq 2000 sequencer according to standard Illumina protocols. Samples were sequenced to a depth of approximately 29 million reads per sample. Reads were aligned to the rn4 rat genome assembly using the GSNAP program[50], version 2012-07-20.

Differential expression analysis

Expression quantification was performed using the Cufflinks program and differential expression analysis was performed using the CuffDiff program[51] version 2.2.1, using the Ensembl database[52] version 69 rat transcriptome annotation. Cufflinks quantifies gene expression as Fragments per Kilobase per Million mapped reads (FPKM), which corrects for different transcript lengths and sample sequencing depths. These expression values are further normalized by CuffDiff across the samples in the experiment by scaling them according to the geometric mean of the samples. Based on these expression data, CuffDiff calculates differentially expressed genes between all pair-wise combinations of conditions (rat mPFC age in PNDs), taking replicates per condition into account and using a multiple testing-adjusted p-value of $p<0.05$. As CuffDiff uses sophisticated statistical models to calculate differentially regulated genes, no fold change or number of reads were required as cut-off to detect differentially regulated genes. Two outlier samples (WT14 21-3, WT21 16-2) were excluded in the differential expression analysis. Clustering was performed using the Multi-Experiment Viewer[53] version 4.9. Gene Ontology analysis was performed using the DAVID website[54] and PANTHER[55].

Long intergenic non-coding RNA (lincRNA) analysis

Annotated lincRNAs were taken from the Ensembl database version 82 (rn6 rat genome assembly), and mapped to the rn4 rat genome assembly using the lift-over tool of the UCSC Genome Browser database[56] due to the lack of lincRNA annotation in the Ensembl database version 69 (rn4 assembly). Only 2305 of the 3023 annotated lincRNAs that could be completely transferred to the rn4 assembly were used. Differentially expressed lincRNAs were determined using the Cuffdiff program with an adjusted p-value of $p<0.05$. The set includes all lincRNAs that are significantly differentially expressed between any two time points. Protein-coding genes were taken from the Ensembl 69 (rn4) annotation, and the nearest genes to the lincRNAs were determined using the "closestBed" command of the BEDTools software suite[57] version 2.25.0. Correlation analysis and plotting were done using the R statistical software package (R Core Team (2015) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria; URL: http://www.R-project.org/). Gene ontology analysis of the set of closest protein-coding genes was performed using the DAVID website[54]. Human orthologs of rat lincRNA-neighboring protein-coding genes were obtained using the Ensembl BioMart webtool[58], using Ensembl database version 85. Nearest human genes to these protein-coding genes were mapped using the "closestBed" command using gene locations obtained from Ensembl BioMart (database version 85). Classification of these nearest genes into protein-coding or lincRNA genes was based on the Ensembl "biotype" annotation of the genes.

Alternative splicing analysis

Alternative splicing analysis was performed using the DEXSeq[59] package (version 1.14.2) of the R Bioconductor Statistical Software Suite[60]. This software package identifies alternatively expressed exons after normalization for differences in total gene expression levels. The RGSC3.4 (rn4) rat transcriptome from Ensembl database[52] version 69 was used for this analysis. Mapping of rat alternatively spliced exons to the hg19 human genome assembly was done using the UCSC genome browser[56] lift-over tool, and overlap with human exons from the GENCODE[61] version 19 annotation was done using the BEDTools "intersectBed" command.

Neuropsychiatric GWAS gene enrichment analysis

Genes associated with neuropsychiatric genome-wide association studies (GWAS) were obtained from the NHGRI-EBI GWAS Catalog[62] (http://www.ebi.ac.uk/gwas/), spreadsheet version v1.0.1, downloaded on 22 August 2016. Genes were filtered using the GWAS Catalog trait ontology terms: "autism" (optionally followed by other words, e.g. "autism spectrum disorder"), "schizophrenia", "bipolar depression", "unipolar depression" and "attention deficit hyperactivity disorder". GWASs combining multiple of these neuropsychiatric disorders were excluded. Ensembl BioMart was used to map the human GWAS genes to rat orthologs. For the enrichment analyses, rat cluster genes were first filtered for those that had human

orthologs according to Ensembl BioMart (database version 85).

Quantitative Reverse Transcription PCR

For validation of the RNA-seq, one gene per cluster (from k-means clustering) was selected for quantitative reverse transcription PCR (RT-qPCR). Primers were designed using Primer3 online software (http://frodo.wi.mit.edu). See Supplementary Table 1 for primer sequences. Complementary DNA (cDNA) was synthesized using 500 ng of total RNA in a reverse transcription reaction using iScript cDNA Synthesis Kit according to manufacturer's protocol (Bio-Rad, Veenendaal, The Netherlands). For the cDNA reaction we pooled five rat PFC samples for each time point. qPCR reactions were performed in a 7500 Fast Real Time PCR System (Applied Biosystems, Foster City, CA, U.S.A) using the SYBR Green fluorescence quantification system (GoTaq® qPCR Master Mix, Promega Benelux b.v. Leiden, The Netherlands). Thermal cycling was initiated with incubation at 95°C for 10 min followed by 40 cycles of 95°C for 30 sec and 60°C for 1 min. To normalize the cDNA content of the samples, we used the comparative threshold cycle (CT) method[63], which consists of the normalization of the number of target gene copies versus the average of two endogenous reference genes, *Gabbr1 and Kif5c* (stable genes in the RNA-seq dataset throughout all ages based on the Coefficient of Variation (CV = stddev/mean)).

Results

Differentially expressed coding genes

We surveyed the gene expression changes that occured in the mPFC during rat postnatal development. Transcriptome analysis (RNA-seq) was performed in mPFC tissue of male Wistar rats sacrificed by decapitation at five different time points; PND8, PND14, PND21, PND35 and PND70. We performed pair-wise comparisons across all time points and observed a total of 10336 differentially expressed genes at a multiple testing-adjusted p-value of $p<0.05$ using CuffDiff[51] and the rat transcriptome from the Ensembl database[52]. More than 5000 genes are differentially regulated when gene expression is compared between PND8 and PND14 (2638 and 2447, down- and up-regulated, respectively). This is the largest change across all comparisons between successive time points, and a continuous decrease in the number of differentially expressed genes with age was evident (Figure 1 and Online Supplementary Table 2). This suggests that the majority of the postnatal expression changes between successive time points take place in the first three postnatal weeks. The numbers of up- and down-regulated genes are similar for all pair-wise comparisons (Supplementary Table 3).

Next, we investigated the functions of the differentially expressed genes between successive time points with gene ontology (GO) analysis[64] using the NIH DAVID website[54] (Figure 1 and Online Supplementary Table 2). This analysis revealed that **cell cycle** (e.g. cyclin related genes, such as *Ccna2* and *Cdk4*) and **translational elongation** genes (e.g. ribosomal proteins, such as *Rpl18* and *Rpl4*) were significantly down-regulated during early development from PND8 to PND14 and from PND14 to PND21, while genes linked to **transmission of nerve**

impulse (e.g. neurotransmitter receptors, such as *Grin2a* and *Gabrd*) were up-regulated in the same developmental period. In addition, genes linked to **myelination** (e.g. *Cldn11* and *Mbp*) are up-regulated from PND14 to PND21. Between PND21 and PND35, genes linked to **cell adhesion** (e.g. cadherins, such as *Cdh5* and *Pcdha13*), **vasculature development** (e.g. collagens, such as *Col1a1* and *Col1a2*) and **cell migration** (e.g. *Reln* and *Dcx*) were down-regulated while genes linked to **translational elongation** (e.g. ribosomal proteins, such as *Rps29* and *Rpl5*) were up-regulated. From adolescence (PND35) to adulthood (PND70) there was little change in gene expression (Figure 1) and no apparent enriched biological processes were detected in the GO analysis. Of interest, a shift from developmental processes (**cell adhesion, neuron differentiation** and **cell migration**) to maintenance (**translational elongation**, **response to organic substance** and **metabolic processes**) was detected between PND21 and PND35. We confirmed these findings using the alternative PANTHER[55] GO analysis website, including the switch in gene expression from developmental processes to metabolic processes or maintenance during postnatal PFC development (see Online Supplementary Table 2).

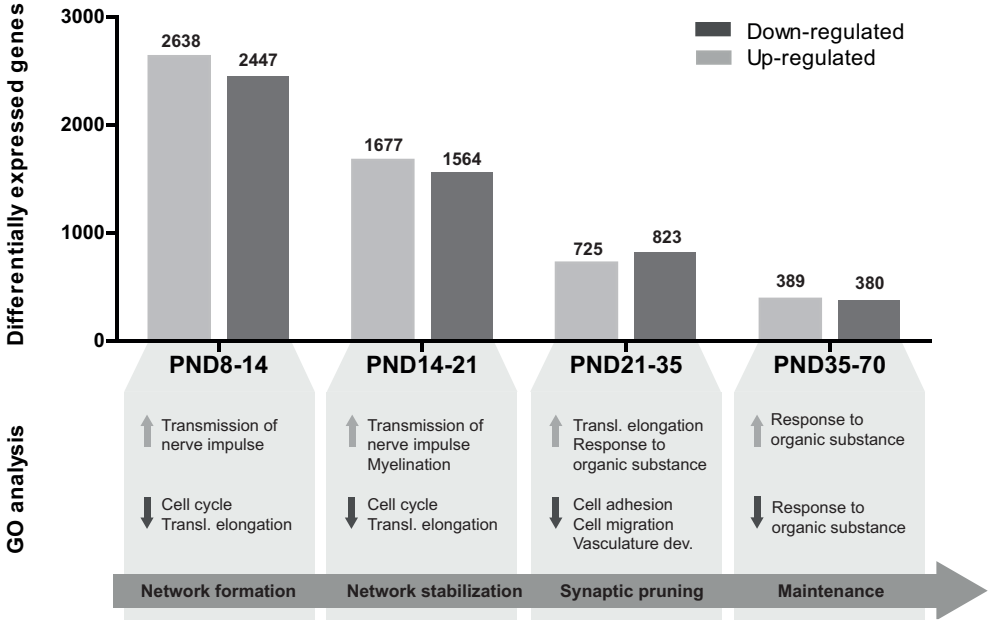


Figure 1. Differential expression analysis between successive time points using RNA-seq. Number of differentially expressed genes (adjusted p<0.05) in the rat medial prefrontal cortex (mPFC) during development is shown in the bar graph. Top gene ontology (GO) terms for up- and down-regulated genes are shown under the graph. Top GO terms fit with mPFC development, as shown by the horizontal grey arrow.

To investigate the overall patterns of gene expression changes during rat mPFC development beyond changes between consecutive time points, we clustered the total set of 10336 genes that changed significantly between any two time points into nine clusters using

k-means clustering (Multi-Experiment Viewer[53]). From these nine clusters several cluster pairs show mirrored patterns, namely clusters A and D, B and E, C and F, and G and H (Figure 2). The mirrored patterns of gene expression suggest that these two gene groups are involved in complementary processes. For validation of the RNA-seq analysis, we performed RT-qPCR for one gene per cluster. The selected genes are linked to the GO-terms mentioned in Figure 2. Our RT-qPCR validation showed that most of these genes have similar expression patterns to the RNA-seq data for all clusters (Supplementary Figure 2). In addition, we compared our gene expression data with human PFC expression data from Jaffe and colleagues[65]. Generally, we observed similar expression patterns for both human and rat genes in all gene clusters (see Supplementary Figure 3). To characterize the functions of the transcripts sharing common expression patterns during development, we performed a GO analysis using the NIH DAVID website on each cluster (Figure 2 and Online Supplementary Table 4). Consistent with the pair-wise comparisons, **cell cycle** genes are enriched in clusters D and E; both clusters show a continuous decrease in expression from the first postnatal week (PND8) until PND21, after which their levels either stabilize (cluster D) or continue to decline into adulthood (cluster E). These two clusters contain for example cyclins (e.g. *Ccna2*) and cyclin-dependent kinase enzymes (e.g. *Cdk4* and *Cdk6*) which control the progression of cells through the cell cycle[66,67]. Additionally, **RNA processing** and, in particular, **translational elongation** genes are enriched in cluster D and H, both of which show a strong decrease in expression in the second postnatal week followed by either stable levels up to adulthood (cluster D) or a slow increase in expression after PND21 (cluster H). These terms, which are associated with protein production rate, are also enriched amongst genes that are down-regulated during the progression from infancy (PND21) to adolescence (PND35) in the consecutive time point comparisons (Figure 1). Furthermore, consistent with the pair-wise comparisons, GO terms related to inter-neuronal communication (**ion transport, transmission of nerve impulse, cell-cell signaling** and **intracellular signaling cascade**) are found in clusters A and G, which show increases in expression level during early development. Both clusters show a strong increase in expression in the second postnatal week, which is followed by either stable levels up to adulthood (cluster A) or a slow decline in expression after PND21 (cluster G). The two clusters contain, amongst others, neurotransmitter receptors, such as *Grin2a* and *Gabrd*, which are involved in the transmission of the neurotransmitters GABA and glutamate[68-70]. **Cell adhesion** genes were up-regulated from PND8 to PND14 and expression gradually decreased after that (cluster F). The decrease in expression is also shown in the consecutive time point comparison. This cluster contains, for example, cadherins, such as *Cdh5* and *Pcdh12,* which play important roles in cell adhesion, forming adherens junctions between cells[71]. In contrast to the previous clusters that show enrichment of GO terms also enriched in the pair-wise comparisons, cluster B, which contains genes that increase gradually from the first postnatal week (PND8) until adulthood, is associated with genes involved in **homeostasis** (especially **ion homeostasis**) and **protein phosphorylation**. Finally, cluster I contained a relatively small set of genes with expression that oscillated in early postnatal development

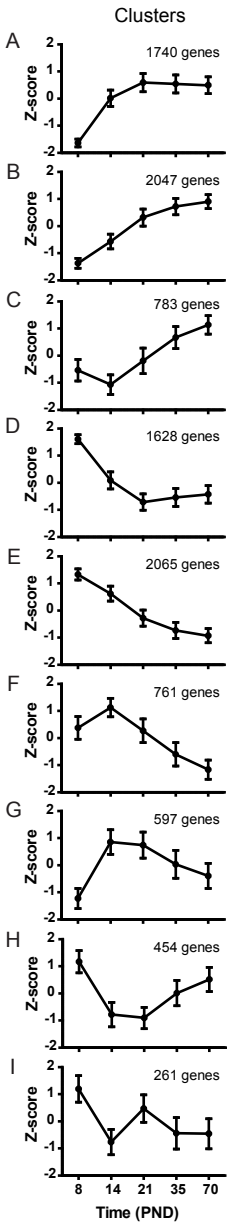
but stabilized in adulthood, which were enriched for **gliogenesis**. This cluster contains for example *Notch1,* a receptor in the Notch signaling pathway, a pathway known to mediate prominent cellular events that result in gliogenesis[72]. We confirmed these DAVID GO analysis findings with the PANTHER GO analysis website, finding similar GO terms for each cluster (Online Supplementary Table 4). In summary, cluster analysis showed that expression of genes involved in **protein phosphorylation**, **interneuronal communication** and **neuronal homeostasis** is increasing during postnatal mPFC development, while expression of genes involved in **cell proliferation** is decreasing during neurodevelopment. Of interest, similar to the pair-wise comparison, the switch from neurodevelopmental processes to maintenance around PND21 was also observed in the cluster analysis. Clusters showing a decrease in expression after PND21 were related to **transmission of nerve impulse** (cluster G) and **cell adhesion** (cluster F), and clusters showing an increase in expression after PND21 were related to **translational elongation** (cluster H) and **homeostasis** (cluster B).

Next, we asked the question whether genes differentially regulated during mPFC postnatal development are relevant for neurodevelopmental and neuropsychiatric disorders. We found that indeed a number of the differentially regulated genes are known to have a function in (pre)frontal cortex development and are associated with disorders, such as intellectual disability, attention-deficit hyperactivity disorder, autism spectrum disorder and schizophrenia (See review by Schubert and colleagues[10]) (Table 1). The majority of these genes showed differential expression at the earliest time points (PND8-14 and PND14-21), which suggests that this early postnatal period is a more sensitive period for the onset of neurodevelopmental disorders compared to adolescence and adulthood.

Furthermore, we examined the enrichment of genes identified by psychiatric GWAS studies in these clusters. Interestingly, we observed that genes with predominantly increasing expression levels during postnatal development (cluster A, B, C, F and G) are enriched for GWAS disease genes (adjusted $p<0.05$, Figure 2; Supplementary Figure 4 and Online Supplementary Table 5). These genes are associated with neurodevelopment-related processes (Clusters A, B, C, F & G), with different clusters being associated with different disorders (Supplementary Figure 4). Psychiatric GWAS genes are not enriched in gene clusters associated with the cell cycle or RNA processing (clusters D, E and H; Figure 2 and Supplementary Figure 4).

Long intergenic non-coding RNAs

LincRNAs have been implicated in the regulation of genes in their genomic vicinity[38] and they have been shown to be important in the dynamic nervous system. We therefore evaluated the expression patterns of annotated lincRNAs during mPFC development. Differential expression of lincRNAs across development was determined using the CuffDiff program[51]. We identified 137 of the 2305 lincRNAs that showed differential expression (adjusted $p<0.05$) between any two time points across development (Online Supplementary Table 6). This proportion (6%) is lower than for the differentially expressed protein-coding genes (30%). This is likely, in part, due to the generally lower overall expression levels of lincRNAs (Figure 3A), which would



GO term	Benjamini	Example genes	GWAS genes
Ion transport	4.10E-07	*Slc17a7, Kcnh5, Gabra1*	3.8
Intracellular signaling cascade	2.49E-05	*Mapk1, Prkcb, Htr2a*	
Transmission of nerve impulse	3.72E-05	*Grin2a, Gabrd, Grm2*	
Response to organic substance	6.02E-04	*Adcy2, Tlr4, Pld1*	1.4
Protein phosphorylation	4.10E-03	*Pink1, Tlk1, Tgfb1*	
Homeostasis	4.56E-03	*Cldn1, Car2, Apoe*	
			2.7
RNA processing	1.81E-16	*Prpf4b, Rbm3, Rpl11*	0.4
Cell cycle	1.54E-09	*Cdk6, Kifc1, Sept2*	
Cell cycle	9.41E-12	*Jub, Ccna2, Cdk4*	1.0
Regulation of protein ubiquitination	8.40E-08	*Fzr1, Psma7, Fem1b*	
Cell adhesion	1.72E-03	*Robo2, Cdh5, Pcdh12*	1.7
Transmission of nerve impulse	2.82E-04	*Clstn3, Th, Grik4*	1.5
Oxidative phosphorylation	2.89E-04	*Atp5d, CycS, Snca*	
Cell-cell signaling	3.33E-04	*Grin2b, Shc3, Dlg2*	
Behavior	3.34E-04	*Slc6a1, Vdac1, Crhr1*	
RNA processing	3.19E-08	*Rpl5, Rpl26, Cpsf6*	0.1
Response to progesterone stimulus	2.49E-02	*Txnip, Cav1, Socs3*	0.4
Pos. regulation of dev. process	2.54E-02	*Jag1, Tgfb2, Notch1*	
Gliogenesis	3.65E-02	*Notch1, Znf488, Sox10*	

Figure 2. K-means clustering analysis of differentially expressed genes. Differentially expressed genes were clustered in nine clusters. Graphs depict Z-score of the log-transformed FPKMs (fragments per kilobase per million mapped reads) per cluster against time (postnatal day, PND). Error bars indicate the standard deviation. The number of genes in the clusters is shown in the graphs. Top gene ontology (GO) terms are shown per cluster and p-values (corrected for multiple hypothesis testing using the Benjamini-Hochberg method) show significance level for the enrichment of the GO term. In the figure similar GO terms are grouped into one "umbrella term", for example, synaptic transmission belongs to transmission of nerve impulse and translational elongation belongs to RNA processing. For each GO term three example genes are shown. The last column shows enrichment of neuropsychiatric disorder (autism spectrum disorder, schizophrenia, depression, and attention deficit hyperactivity disorder) GWAS genes per cluster. It shows the -log10 transformation of the multiple testing-corrected p-value (adjusted p-value). Adjusted p-value of 0.05 corresponds to 1.3 after -log10 transformation.

Table 1. Differentially expressed coding genes involved in prefrontal cortex development and associated with neurodevelopmental diseases

Gene	Diff. expr.	Cluster	Involvement in PFC development	Neurodevelopmental disease	References
Proliferation, differentiation and migration of PFC neurons					
Fgf2	PND14-21 Up	B	Involved in production of glutamatergic pyramidal neurons.	AD(H)D: Fgf2 knockout mice show hyperactivity. Schizophrenia: Serum FGF2 levels are increased in people with schizophrenia.	73-75
Fgfr1	PND14-21 Up	B	Required for the proper number of glutamatergic pyramidal neurons.	ID: FGFR1 mutations are responsible for Hartsfield syndrome. AD(H)D: Dysfunctional Fgfr1 signaling is associated with spontaneous hyperactivity. Schizophrenia: FGFR1 levels are higher in schizophrenia and th-fgfr1(tk-) transgenic mice exhibit behavior resembling human schizophrenia.	76-79
Fgfr2	PND8-14 Up	B	Involved in generating excitatory glutamatergic neurons	ID: Mutations in FGFR2 cause Crouzon's or Apert syndrome, which can be associated with MR. AD(H)D: Some Fgfr2 deficient mice display hyperactive behavior. ASD: Deletions of FGFR2 are associated with ASD. Schizophrenia: A SNP flanking the FGFR2 gene is associated with schizophrenia.	80-85
Gad1	PND8-14 Up, PND14-21 Up	B	Regulates migration of GABA-ergic interneurons .	ASD: Gad1 is an ASD susceptibility gene. Schizophrenia: GAD1 expression is altered in schizophrenia patients and is considered a risk gene.	86-89
Tbr1	PND14-21 Up	C	Required for the implementation of regional and laminar identity in post-mitotic neurons.	ASD: TBR1 mutations are found in sporadic autism spectrum disorder.	90,91
Axon guidance, target selection and synapse formation of PFC neurons					
Erbb4	PND8-14 Down	H	Regulates dendritic spine formation and density of PV+interneurons.	ID: ERBB4 is associated with ID. Schizophrenia: Numerous studies implicate ERBB4 as schizophrenia risk gene.	92-96
Eif4e	PND8-14 Down PND21-35 Up	H	Has a role in synaptic function, dendritic spine density and synaptic plasticity of PFC neurons.	ASD: EIF4E shows genetic association with autism. Eif4e transgenic mice display autism-like behaviors.	97-100
Nrp2	PND8-14 Down PND35-70 Up	D	Regulating axon guidance of PFC neurons.	ASD: NRP2 mutations are associated with autism.	101-103
Reln	PND8-14 Up PND14-21 Down PND21-35 Down	F	Regulating spine density and network formation.	ID: Disruption of RELN is associated with MR. ASD: RELN shows genetic association with autism. Schizophrenia: RELN shows genetic association with schizophrenia.	104-109

Snap25	PND8-14 Up PND14-21 Up PND21-35 Up	B	Involved in axonal extension in developing neurons and reduced expression is linked to impaired PFC functioning.	AD(H)D: SNAP25 polymorphisms are associated with AD(H)D. ASD: Association between SNAP25 gene polymorphisms and cognition in autism. Schizophrenia: SNPs in SNAP25 represented a common risk factor of schizophrenia.	110-115
Kalrn	PND8-14 Up PND14-21 Up	A	Involved in nerve growth and axonal development and loss of Kalrn strongly correlates with spine loss in layer 3 PFC neurons.	Schizophrenia: Expression of KALRN mRNA was reduced in the DLPFC of individuals with schizophrenia. AD(H)D: Kalrn allelic variant is associated with vulnerability to adult AD(H)D.	116-118
PFC connectivity					
Disc1	PND14-21 Down	E	Associated with dendritic abnormalities and affected cAMP signalling and hampers the mesocortical dopaminergic network formation.	AD(H)D: DISC1 shows genetic association with AD(H)D in adults. ASD: DISC1 shows genetic association with autism. Schizophrenia: DISC1 is a strong candidate gene for schizophrenia.	119-125
Mapt	PND8-14 Down PND14-21 Down	D	Mutations in MAPT are associated with altered functional connectivity in the human PFC.	ID: MAPT CNVs and microdeletions in patients with ID.	126-129
Slc6a4	PND8-14 Down	E	Involved in proper raphe-prefrontal network formation.	Schizophrenia: Slc6a4 is associated with schizophrenia.	130-132

Abbreviations: AD(H)D, attention deficit hyperactivity disorder; ASD, autism spectrum disorder; ID: intellectual disability; PFC: prefrontal cortex; PV^+, parvalbumin$^+$; CNVs: copy number variations. Synopsis of the most cited genes that have been directly linked to one or more of the developmental events of PFC development (indicated in bold italics) and that have been directly genetically linked to the etiology of ID, ASDs, AD(H)D and/or schizophrenia (based on Table 1 from Schubert and colleagues[10] and further literature search). Notes: (1) A selection of references was made when more than two references per disease were found; (2) Focus was on only those genes that were proven to be involved in prefrontal developmental events and not just expressed or involved in cortical development in general.

make it more difficult to detect significant differences. As lincRNA expression has been found to be correlated to that of protein-coding genes in their genomic vicinity during mouse brain development[133], we investigated the degree of expression correlation between lincRNAs and their nearest protein-coding genes during rat mPFC development. We found that, although the correlation is not clear for the full set of 2305 lincRNAs (Figure 3B), there is a clear positive correlation for the expression of the identified 137 differentially expressed lincRNAs and their neighboring protein-coding genes (Figure 3C). As lincRNAs are poorly annotated with respect to function, and given that they are frequently involved in the regulation of neighboring genes, we performed a GO analysis using the NIH DAVID website on the nearest protein-coding genes. Using the nearest protein-coding genes to all 2305 lincRNAs, GO analysis showed an enrichment of GO terms related to **neuron differentiation/cell projection** as well as **positive regulation of gene expression** (Table 2, Online Supplementary Table 6). R**egulation of transcription** and some terms related to development (e.g. **positive regulation of cell differentiation** and **morphogenesis of a branching structure**) were still present in the subset of 137 differentially expressed lincRNAs, although not significantly enriched (using Benjamini correction), probably due in part to the small number of genes (Online Supplementary Table 6). Comparison of the 10336 differentially expressed coding genes with the 137 neighboring genes of the differentially expressed lincRNAs resulted in 71 coding gene-lincRNA pairs with both differentially expressed coding gene and differentially expressed lincRNA. Of these 71 pairs, 32 pairs showed a strong correlation between coding genes and lincRNA expression (Pearson correlation (PCC) >0.75) (Online Supplementary Table 6). Strongly correlated differentially expressed coding gene-lincRNA pairs were present in almost all clusters, except cluster H. Among the 32 strongly correlated coding gene-lincRNA pairs, several coding genes are linked to **neurodevelopment** (e.g. *Wnt5a*[134,135], *Actr3b*[136] and *Cbln1*[137]) and to **regulation of gene expression** (e.g. *Khdrbs2*[138], *Abra*[139] and *Fos*[140]) (Figure 4). Most of the 32 strongly correlated lincRNA-neighboring gene pairs have an expression pattern that is linked to cluster A (n=10), B (n=8) and E (n=6), based on the coding genes (Online Supplementary Table 6). The enrichment of lincRNA-coding gene pairs in these clusters (which are also the largest coding gene clusters) suggests that, based on the cluster GO terms, most of these pairs are involved in neurodevelopment, homeostasis or cell cycle. Expression of lincRNA-coding gene pairs linked to neurodevelopment (cluster A) increases during the early postnatal period and becomes stable after PND21, while expression of lincRNA-neighboring gene pairs involved in homeostasis (cluster B) continues to increase after PND21. In addition, expression of lincRNA-neighboring gene pairs involved in cell cycle processes (cluster E) decreases with time, suggesting reduced proliferation. These GO annotation patterns for the lincRNA-neighboring protein-coding genes suggest that around PND21 homeostatic processes start to exceed developmental processes. Given that lincRNAs are frequently involved in the regulation of neighboring genes, it is likely that the differentially expressed lincRNAs identified in this study are also important for this switch from development to maintenance.

To examine whether these rat lincRNAs are conserved in humans, we sought to

identify their corresponding human orthologs based on sequence similarity, but none of the 2305 rat lincRNAs could be mapped to human lincRNAs. It is known that lincRNA sequences are poorly conserved across vertebrates, but that their genomic locations are more often retained[141]. We therefore compared the rat protein-coding genes neighboring these lincRNAs to their human orthologs to see if these also had neighboring lincRNAs. We found that 24.6% (375 out of 1527) of these human protein-coding orthologs did indeed have lincRNAs as their closest neighboring genes (Online Supplementary Table 6), indicating a higher level of conservation of lincRNA genomic location than sequence between the rat and human genomes.

Taken together, a subset of lincRNAs show differential expression during mPFC development which correlate with the expression of neighboring neurodevelopmental protein-coding genes, suggesting that these lincRNAs are involved in the regulation of neurodevelopmental gene expression and thereby indirectly contribute to shaping the developing brain.

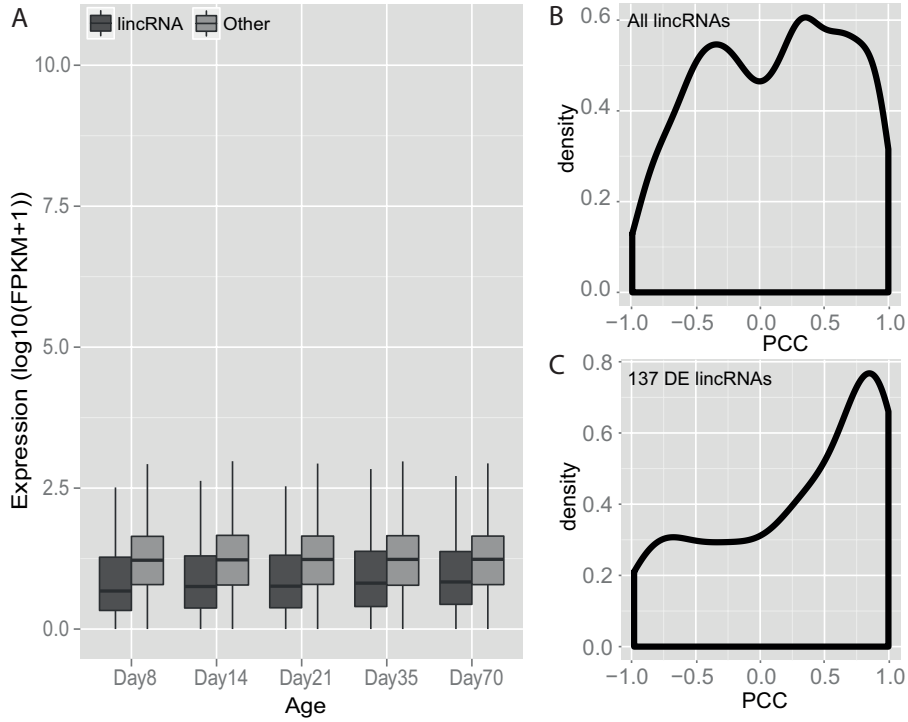


Figure 3. Expression of lincRNA in the medial prefrontal cortex of the rat. (A) LincRNAs showed overall lower expression compared with coding genes on all five time points. Expression level is depicted as log10-transformed FPKM values (fragments per kilobase per million mapped reads) after adding a pseudocount of 0.01 to avoid infinite values. (B) Correlation between lincRNA expression and nearest coding gene expression using Pearson correlation for all 2305 expressed lincRNAs. (C) Correlation between lincRNA expression and nearest coding gene expression using Pearson correlation for the 137 differentially expressed lincRNAs. The 137 differentially expressed lincRNAs showed a clear positive correlation with the expression of their neighboring coding genes. PCC = Pearson Correlation Coefficient, DE = differentially expressed.

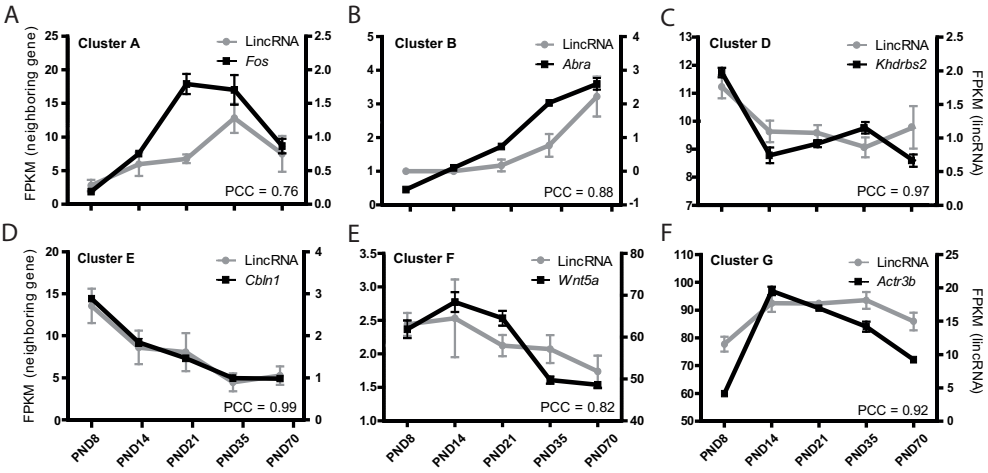


Figure 4. Examples of coding gene-lincRNA pairs in the medial prefrontal cortex. Examples of coding gene-lincRNA pair expression (FPKM) with Pearson correlation coefficient (PCC) >0.75. (A) Example cluster A; *Fos* and ENSRNOG00000057609 expression. (B) Example cluster B; *Abra* and ENSRNOG00000054328 expression. (C) Example cluster D; *Khdrbs2* and ENSRNOG00000055308 expression. (D) Example cluster E; *Cbln1* and ENSRNOG00000054562 expression. (E) Example cluster F; *Wnt5a* and ENSRNOG00000058571 expression. (F) Example cluster G; *Actr3b* and ENSRNOG00000061925 expression.

Table 2. Top 10 gene ontology terms for all lincRNAs expressed in the mPFC

GO term	Count	Benjamini	Example genes
Neuron differentiation	81	7.71E-13	*Cxcr4, Nrp1, Drd1a, Nrcam, Ntrk3*
Positive regulation of gene expression	83	2.21E-09	*Fos, Abra, Shh, Runx2, Tcfap2b*
Regulation of transcription from RNA polymerase II promoter	87	1.05E-08	*Fos, Abra, Shh, Runx2, Tcfap2b*
Positive regulation of transcription	80	1.21E-08	*Fos, Abra, Shh, Runx2, Tcfap2b*
Sensory organ development	49	1.71E-08	*Ntrk2, Edn1, Nox3, Neurod1, Maf*
Positive regulation of macromolecule metabolic process	106	4.30E-08	*Fos, Abra, Dlx2, Egr2, Ntf3*
Regulation of cell proliferation	91	5.45E-08	*Htr2a, Igf1, Klf5, Sox2, Ptprm*
Positive regulation of transcription from RNA polymerase II promoter	63	7.45E-08	*Fos, Abra, Shh, Runx2, Tcfap2b*
Positive regulation of macromolecule biosynthetic process	87	8.63E-08	*Fos, Abra, Shh, Runx2, Tcfap2b*
Positive regulation of transcription, DNA-dependent	70	1.11E-07	*Fos, Abra, Shh, Runx2, Tcfap2b*

Alternative exon usage

Alternative exon usage is a process that occurs extensively in the nervous system[142]. We therefore surveyed alternative exon usage during rat mPFC development using the DEXSeq package[59] of the R Bioconductor Statistical Software Suite[60], which detects differentially expressed exons after correcting for differences in overall gene expression. At an adjusted p-value of $p<0.05$, we identified 1634 differentially expressed exons in 796 genes. Of these genes, 491 also showed differential gene expression across development. Functional analysis of the 796 genes using the DAVID website[54] showed an enrichment of GO terms related to **neuron differentiation/projection**, **neurotransmission** and **cytoskeleton organisation** and the **axon guidance** pathway (Table 3 and Online Supplementary Table 7).

Of the genes with alternative exon usage, 166 showed differential expression of 5' exons, and therefore differential promoter usage (Online Supplementary Table 7). A striking example is the protocadherin alpha gene cluster (*Pcdha13*), which is involved in the establishment of cell-cell connections and neuronal wiring in the brain[143,144]. This cluster has multiple alternative promoters that share the same 3' exons, resulting in a set of transcript isoforms that differ only in their 5' exons[145]. These alternative 5' exons encode variants of the extracellular region of this neurodevelopmental transmembrane protein, while the common 3' exons encode the common intracellular region[143,146]. For this gene cluster, the overall expression level decreases during mPFC development, but exons transcribed from the earlier promoters (exons E01, E02, E07), associated with longer genomic isoforms, are more strongly affected than those transcribed from the proximally located promoters (exons E11, E12, E13), leading to a quantitative shift in favor of the genomically shorter isoforms (Figure 5A). 585 of the 796 genes with alternative axon usage showed alternative splicing in internal exons (cassette exons or mutually exclusive exons). An interesting example of a gene with cassette exons is the neurotransmission-related gene synaptosomal-associated protein 25 (*Snap25*). Two isoforms (isoforms A and B) exist, with nine amino acid residue differences between the two isoforms, including a re-localization of one of the four cysteine residues[147]. There is a switch from isoform A (incorporating exon E4) to isoform B (incorporating exon E5) during development (Figure 5B), which has also been shown in mice[110]. 252 genes harbored alternatively spliced 3' exons. An example is *Srgap2* (although the first 3' exon does not reach the threshold of significance), which is involved in axon guidance. The shorter splice variants are more highly expressed in the later developmental stages (Supplementary Figure 5). It should be noted that the group of alternatively spliced internal exons might also contain genes with alternative promoter usage, since a promoter exon can also be an internal exon if it is an inner promoter; additionally, some of the alternative 3' or 5' exons might have ended up in the group of genes with internal exons if the first or last exon was not above the significance threshold.

Interestingly, the majority (1555 out of 1634, or 95%) of these alternatively spliced exons overlap with exon annotation in the human genome (Online Supplementary Table 7). Therefore, these exons could also be alternatively spliced in human PFC development. Indeed,

comparison with 24 genes reported by a previous study to be alternatively spliced during human PFC development[48] shows a statistically significant overlap with our rat alternatively spliced genes, despite different experimental and analytical methodologies (9 out of 24 genes are present in our 796 alternatively spliced genes; $p = 1.3 \times 10^{-8}$, hypergeometric distribution test).

In summary, alternative exon usage occurs in neurodevelopmental genes during rat PFC development, suggesting that dynamic exon usage may be important for proper brain development.

Table 3. Top 10 gene ontology terms alternative exon usage in the mPFC

GO category	GO term	Count	Benjamini	Example genes
Biological process	Cell projection organization	42	3.27E-07	*Snap25, Ctnna2, Robo2, Ncam2, Cdh23*
	Transmission of nerve impulse	37	3.64E-07	*Snap25, Grik5, Grm5, Syn2, slc17a7*
	Neuron differentiation	47	7.12E-07	*Snap25, Ctnna2, Robo2, Ncam2, Cdh23*
	Cellular component morphogenesis	42	7.55E-07	*Snap25, Ctnna2, Robo2, Ncam2, Cdh23*
	Cell morphogenesis	38	3.29E-06	*Snap25, Ctnna2, Robo2, Ncam2, Cdh23*
	Neuron development	38	4.75E-06	*Snap25, Ctnna2, Robo2, Ncam2, Cdh23*
	Synaptic transmission	29	5.44E-06	*Snap25, Grik5, Grm5, Syn2, slc17a7*
	Regulation of cytoskeleton organization	20	6.98E-06	*Dst, Map4, Mapt, Rock2, Tpm1*
	Regulation of organelle organization	25	8.41E-06	*Dst, Map4, Mapt, Rock2, Tpm1*
	Neuron projection development	31	3.73E-05	*Snap25, Dcx, Robo2, Ncam2, Vcan*
KEGG_PATHWAY	Axon guidance	15	3.91E-02	*Srgap2, Robo2, Rock2, Cxcl12, Pak1*

Discussion

We provide a comprehensive resource of gene expression in the rat developing mPFC from infancy to adulthood, including differentially expressed protein-coding genes, lincRNAs and alternative exon usage in protein-coding genes. We show that most transcriptional changes occur in the first three postnatal weeks and the number of differentially regulated genes reduces toward adulthood. Besides providing a comprehensive resource, we describe expression patterns of different functional clusters of coding genes during mPFC development. We detected an expression pattern switch from genes involved in neuronal network establishment in infancy to those involved in neuronal homeostasis and maintenance in adulthood. This genetic switch is likely regulated by several parallel and/or complementary mechanisms including dynamic expression of protein-coding genes, lincRNAs and alternative exons. In addition, we show that several differentially regulated genes are known to be involved in neurodevelopmental and neuropsychiatric disorders and that specific clusters of genes are enriched for GWAS genes that are associated with developmental and neuropsychiatric

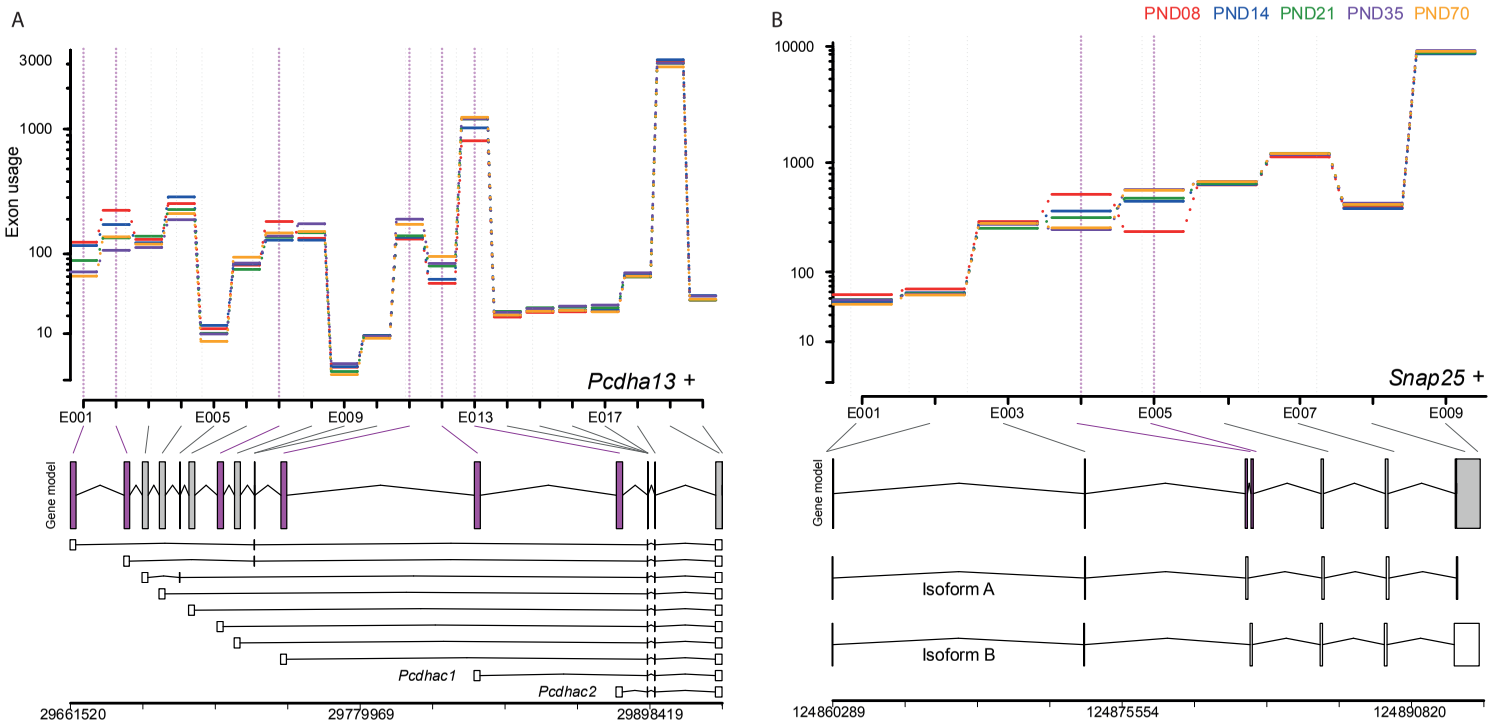


Figure 5. Alternative splicing in the medial prefrontal cortex of the rat. Graphs show expression per exon corrected for overall expression level between time points. Colours represent the different time points during development in postnatal days (PND). Vertical purple dashed lines depict differentially expressed exons during development, caused by alternative exon usage. The upper x-axis depicts exon numbers. The y-axis shows normalized counts per exon after correcting for total transcript expression differences between the time points. The different isoforms are shown under the graph. The first one (gene model) represents the summary of all isoforms and the different isoforms are shown below. Differentially expressed exons are shown in purple. The lower x-axis (under the isoforms) shows the genomic location of the isoform. + or- behind the gene name represents the strand on which the gene is located. Two examples of splicing are shown. (A) Differential expression of 5' exons (differential promoter usage). Example is the protocadherin alpha gene cluster (*Pcdha13*), which has multiple alternative promoters that share the same 3' exons. Exons E11, E12, E13 show higher expression at PND70 (orange) compared to earlier time points. Exons E01, E02, E07 show higher expression at earlier stages (PND8, red and PND14, blue) compared to adolescence and adulthood. (B) Alternative splicing in internal exons (cassette exons or mutually exclusive exons). Example of a gene with casette exons is the neurotransmission related gene synaptosomal-associated protein 25 (*Snap25*). Two isoforms exist and there is a switch from isoform A (incorporating exon E4) to isoform B (incorporating exon E5) during development.

diseases, suggesting that the catalog of gene expression we generated in this study can also be used as a resource to study such disorders.

The gene expression patterns found in our rat mPFC developmental time course show substantial overlap with the gene expression patterns observed by Semeralul and colleagues[41], who studied mouse PFC development at five time points (2, 3, 4, 5 and 10 weeks old) using gene expression microarrays and found that the majority of expression changes occurred between postnatal weeks 2 and 4. This indicates that expression patterns of important pathways are highly conserved between rodents and therefore it is likely also conserved in different strains of rodents. Furthermore, in microarray studies of human[39,148] and non-human primates[43], gene expression changes in PFC or neocortex are shown to be most obvious from birth through infancy, which is consistent with our RNA-seq time course data (Figure 1). In a human RNA-seq study of PFCs across six life stages[65], gene expression generally showed similar patterns as in our rat study (Supplementary Figure 3), although the human expression data are noisier than the rat data (larger standard errors for the z-scores). The more homogeneous genetic background and more precisely synchronized ages of our rat mPFCs relative to the human post-mortem PFCs probably contributes to the difference in variability levels between our rat and the human RNA-seq data. Taken together, our rat mPFC RNA-seq analyses corroborate previous studies in different species of PFC gene expression during postnatal development, and highlight the conservation of gene expression dynamics during PFC postnatal development from rodents to humans.

Our GO analysis of the differentially expressed coding genes between successive time points and of the genes clustered in the k-means clustering showed several GO terms that match with neurodevelopmental processes taking place during PFC development, and with findings from other studies. The decrease in expression of genes involved in the **cell cycle** (PND8-14, PND14-21, cluster D and E), as also described in mice by Semeralul and colleagues[41], is consistent with the decrease in production of new neurons and supportive cells as brain growth slows down and the brain matures. Mature neurons and supportive cells will enter a postmitotic state, which is in line with the decrease in expression of cell cycle control genes. Increased expression of genes involved in inter-neuronal communication (**ion transport**, **transmission of nerve impulse**, **cell-cell signaling and intracellular signaling cascade**, PND8-14, PND14-21, cluster A and G) coincides with the explosion of synapse formation during early brain development, known as exuberant synaptogenesis, which is important for the formation of brain networks[11]. Ion transport by ion channels plays an important role in intercellular communication. Ion transport is essential for the regulation of action potentials[149]. When an action potential arrives at the axon terminal, synaptic transmission occurs by the release of neurotransmitters, which bind to receptors on the postsynaptic neuron and activate downstream intracellular signaling cascades. **Cell adhesion** genes were up-regulated from PND8 to PND14 and expression gradually decreased after that (PND21-35, cluster F). The increase in expression may reflect synapse formation and ongoing migration of cells in early postnatal development that is guided by adhesion molecules[150].

The decrease in expression of cell adhesion genes starting from PND14 was also found by Semeralul and colleagues[41] in mouse PFC tissue and may reflect specification of neuronal networks by elimination of exuberant undesired connections, a process known as synaptic pruning. Synaptic pruning is a process that is strongest around adolescence in both rats and humans[11,151]. In rats it occurs around PND30 in the PFC[11] and a recent paper demonstrated that effective axon pruning requires destabilization of cell adhesion[152]. However, there are also studies in primates showing that synaptic pruning might differ between layers[153] and studies reporting an earlier peak of synaptic density around birth and only a slight though significant decline in synapses by pruning till 20 years of age[154]. The up-regulation of **myelination** genes from PND14 to PND21 as rats approach adolescence may reflect the stabilization of synaptic networks after the early period of exuberant synaptogenesis and synaptic remodeling[11,155]. Genes involved in **homeostasis** (cluster B), a state mainly present in the developed adult brain, increased gradually from the first postnatal week (PND8) till adulthood. Furthermore, a strong shift from developmental processes (**cell adhesion, neuron differentiation, cell migration**) to maintenance (**translational elongation**, **response to organic substance**, **glucose metabolism**) is detected between PND21 to PND35. From PND35 to PND70 gene expression changes become less dynamic, as is reflected in the lowest number of differentially expressed genes, suggesting that developmental processes are mostly finished at this stage and maintenance and homeostasis become more important activities in the brain. Adolescence starts around PND35 and is characterized by shaping of cognitive and emotional functions by synaptic pruning[11]; however, we do not see many gene expression changes in the mPFC during this developmental stage. Possibly the gene expression changes that are needed for effective pruning, such as decreasing the expression of cell adhesion genes, take place before adolescence starts. In addition, pruning is highly dependent on environmental factors and more expression changes might occur if rats are exposed to a variety of environmental triggers instead of keeping the environment relatively stable. Finally, it is worth bearing in mind that while this study only focused on postnatal neurodevelopment, there is also a lot of neurodevelopment going on prenatally[10].

The dynamic gene expression profile from infancy to adulthood implies that regulatory events of gene expression are important for the development of neuronal networks and that disturbance of these events (e.g. caused by environmental factors such as stress and drugs) might lead to abnormal functioning, often manifested in neurodevelopmental and neuropsychiatric disorders[156,157]. This is supported by the enrichment of genes associated with neuropsychiatric disorders amongst the genes that change in expression during postnatal rat mPFC development (Figure 2 and Supplementary Figure 4). In particular, genes that increase in expression during early life stages are associated with these disorders, while genes that decrease in expression do not show similar enrichments (Figure 2 and Supplementary Figure 4). The former genes are generally associated with neurodevelopment-related processes while the latter are more associated with cell proliferation and housekeeping processes (Figure 2 and Supplementary Table 4).

Besides protein-coding genes, we also investigated lincRNAs. LincRNAs are involved in regulating the expression of neighboring genes[38], but so far they are poorly annotated (in rodents especially). There is one study describing eight lincRNAs that showed changes in expression during human cortex development; however, these lincRNAs had limited evolutionary conservation and were primate-specific[158]. Our rat mPFC-expressed lincRNAs similarly showed low sequence similarity with human genes, and none could be mapped to human lincRNAs based on sequence similarity. However, a quarter of the human orthologs of the rat protein-coding genes neighboring these lincRNAs were also located adjacent to lincRNAs, suggesting that these genes are also associated with lincRNA regulation in humans. lincRNAs can affect gene regulation in different ways and genomic location and RNA secondary structure are also often important for their function[141]. We found lincRNAs with neighboring genes involved in neurodevelopment showing a strong coexpression (Pearson Correlation Coefficient > 0.75), which suggests that these lincRNAs are involved in the regulation of expression of their neighboring gene and thereby also the regulation of neurodevelopmental processes. An example is lincRNA ENSRNOG00000058571 with its neighboring gene *Wnt5a* (Figure 4E). Both show a small increase in expression from PND8 to PND14 and a decline after PND14, which matches with the role of *Wnt5a* in the regulation of cell migration and adhesion through focal adhesion dynamics[135]. Another example is lincRNA ENSRNOG00000057609 with its neighboring gene *Fos* (Figure 4A), a transcription factor often used as a marker for neuronal activity. Fos knockout mice show impaired brain development[159,160], suggesting that Fos has an as yet undefined role in neurodevelopment. Both, *Fos* and its neighboring lincRNA, show an increase in expression when new synapses are formed (PND8 till PND21) and subsequently a decrease in expression[161]. This is consistent with the switch from neurodevelopment to maintenance. Several studies have shown that lincRNAs can act as enhancer elements, promoting the transcription of their neighboring coding genes[162-164]. lincRNAs are also involved in epigenetic processes by recruiting and regulating chromatin-modifying complexes and in posttranscriptional regulation[34,165]. However, regulating the expression of the nearest protein-coding gene is only one of the functions of lincRNAs. There is also evidence that lincRNAs can work in *trans* rather than in *cis*, by regulating the expression of distant genes[166]. Using these different mechanisms, lincRNAs can affect a variety of processes in neurodevelopment. It will be of interest to functionally test the differentially regulated lincRNAs identified in this study and search for human counterparts. Besides lincRNAs, small non-coding RNAs, such as miRNAs, are also important in brain development[167]. However, we did not further investigate these small non-coding RNAs as our RNA-seq protocol is not suited to their accurate quantification (due to size selection for 300 bp fragments).

Analysis of alternative exon usage resulted in 1634 differentially expressed exons in 796 genes. We found several genes involved in **axon guidance**, **neuron differentiation/ projection**, **neurotransmission** and **cytoskeleton organisation,** which are all processes that are highly important in early brain development. In human PFC development alternatively

spliced genes seem to play roles in neurotransmission and cytoskeleton organization as well[48]. As can be expected for protein-coding genes, there is good conservation between rat and human exons and almost all the rat alternatively spliced exons map to human exon annotation (Online Supplementary Table 7). In addition, a statistically significant 9 of the 24 alternatively spliced genes that were validated in human PFCs[48] are also present in our list of genes with alternative exon usage ($p = 1.3 \times 10^{-8}$, hypergeometric test). This suggests that there is similarity between human and rat alternative splicing during PFC development, although a thorough systematic analysis would be required to confirm this. While it is known that widespread splicing changes occur in brain development[48], the functional implication of differential splicing is unknown for most of the variants. Some genes have been well studied, however, such as the protocadherin alpha gene cluster. This gene cluster is highly expressed during neurodevelopment (peak in first postnatal week), gradually concentrating in the synaptic region, with expression decreasing as the brain matures[168,169]. Chen and colleagues[170] showed that the so-called C-type protocadherin isoforms (*Pcdhac1* and *Pcdhac2,* incorporating exon 12 and 13, respectively), which have different properties compared with the A and B type isoforms, are primarily important for mediating neuronal survival, whereas the role of protocadherins in postnatal developmental processes like neuronal wiring, requires isoform diversity. This suggests that C-type isoforms are more important for maintenance later in life, while others are more involved in developmental processes, such as neuronal wiring, which correlates with our expression data. In our analysis, a higher expression of C-type isoforms is shown later in life (PND35 and PND70) compared with earlier time points, whereas some non-C-type isoforms were higher expressed at the earlier time points (PND8 and PND14) (Figure 5A). Another interesting alternative splicing example is *Snap25,* encoding for synaptosomal-associated protein 25, which is a member of the SNARE complex involved in vesicular release of neurotransmitters. There is a switch from isoform A (incorporating exon E4) to isoform B (incorporating exon E5) during mPFC development (Figure 5B), as shown before in the brain[110,171]. A study using mice overexpressing isoform A showed that the isoform switch alters the facilitation of synaptic transmission. This study suggests that the assembly of different SNARE complexes affects membrane fusion properties and that the developmental switch between *Snap25* isoforms alters the efficacy of synaptic transmission that may contribute to the solidification of developing neural circuitries[172]. The *Srgap2* gene, involved in neuronal migration[173], showed alternative 3' exon usage. In humans, the *SRGAP2* gene has been duplicated three times in the human genome during evolution and one of the shorter variants dimerizes with the longer variant to inhibit its function[174]. It might be that the shorter splice variants in rats (although different from the human duplicates) have a similar function and therefore are expressed higher in later developmental stages during which neuronal migration and neuronal differentiation are less important (Supplementary Figure 5). However, for most genes, it is still unknown what the functions of the different variants are. Further research is needed to unravel the function of all the different transcript variants found during mPFC development in rats.

Changes in the expression of protein-coding genes, lincRNAs and changes in exon usage together facilitate neurodevelopment and the switch to maintenance. The overall expression of protein-coding genes can be affected by both lincRNA regulation and alternative exon usage. *Abra,* encoding for actin-binding Rho activating protein which acts as an activator of serum response factor-dependent transcription, is an example of a gene showing differential expression during development (Cluster B, Online Supplementary Table 4) that might be influenced by alternative exon usage and lincRNA regulation. *Abra* shows alternative exon usage (Online Supplementary Table 7) and is in the close proximity of a differentially expressed lincRNA showing a strong coexpression (Figure 4B). It will be of interest to investigate the function of this gene during postnatal brain development.

Summarizing these results, we find a clear switch in gene expression patterns from regulation of network formation to maintenance around PND21. This switch is probably important for creating a mature PFC directed to the execution of cognitive and emotional tasks that is less susceptible to the environmental factors that shaped the PFC during development. The dynamic gene expression during PFC development shown in this study also emphasizes the developmental stage-specific nature of the expression of many PFC genes, highlighting the importance of taking developmental stage into consideration when studying specific genes involved in PFC development or related diseases.

It should be noted that we used punched mPFC tissue (prelimbic and a little infralimbic) in our analysis, which is a mixture of different cell types. Nevertheless, we could detect gene expression in processes occurring in different cell types of the PFC. For example, from PND14 to 21 we observed an upregulation in expression of genes linked to myelination and genes involved in neurotransmission. Neurotransmission is a process occurring in neurons, while myelin is made by oligodendrocytes. Myelin forms a layer around the axons of neurons and increases the speed of neurotransmission. Our data suggest that from PND14 to PND21 oligodendrocytes are relatively more important than at other time points where neuron-related processes are the major enriched GO-terms associated with differentially regulated genes. However, single cell RNA-sequencing[175] or selecting a specific cell type using fluorescence-activated cell sorting might give more insight into PFC gene expression per cell type. Furthermore, a more comprehensive study involving different brain regions and other tissues would be required to determine whether the expression changes we found are PFC-specific, cortex-specific or brain-specific. Another interesting direction for future research is the investigation of gene expression in layer-specific circuits during postnatal development for example in dorsolateral PFC layer three microcircuits, which are important for working memory and are linked to schizophrenia[176]. Furthermore, it would be interesting to investigate the link between gene expression changes and the development of PFC-dependent behavior, such as social behavior, emotion regulation, decision-making, and attention. The investigation of the functions of different splice variants of neurodevelopmental genes will also give valuable new insights into the role that alternative splicing plays in PFC development.

In conclusion, we provide a data resource of temporal gene expression in the

developing mPFC from infancy to adulthood. We showed that gene expression is dynamic during postnatal development and identified a clear genetic switch from neuronal network establishment in infancy to maintenance in adulthood. This genetic switch is likely controlled by complementary regulatory mechanisms of protein-coding gene expression, lincRNA expression and alternative exon usage. Together with other studies[39-41,48,177], our study makes an important contribution to the effort of completing the 'brain map'. Our multifaceted genome-wide gene expression dataset can be used to study the basic developmental processes of the mPFC and to understand the mechanisms of neurodevelopmental and neuropsychiatric disorders.

RNA-seq datasets

Our RNA-seq datasets can be found online (GEO, GSE79860; NCBI tracking system 17822426).

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Conflict of interest

The authors declare no conflict of interest.

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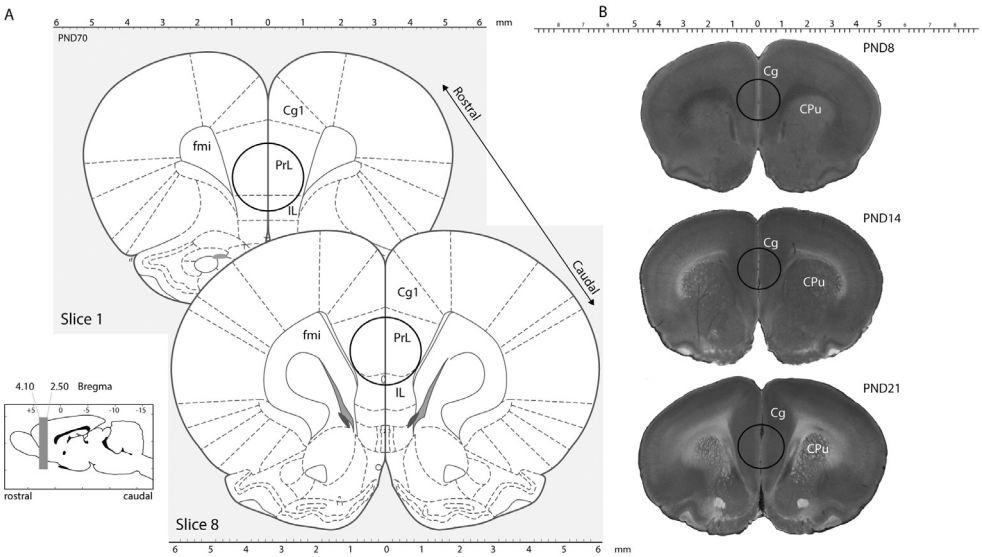
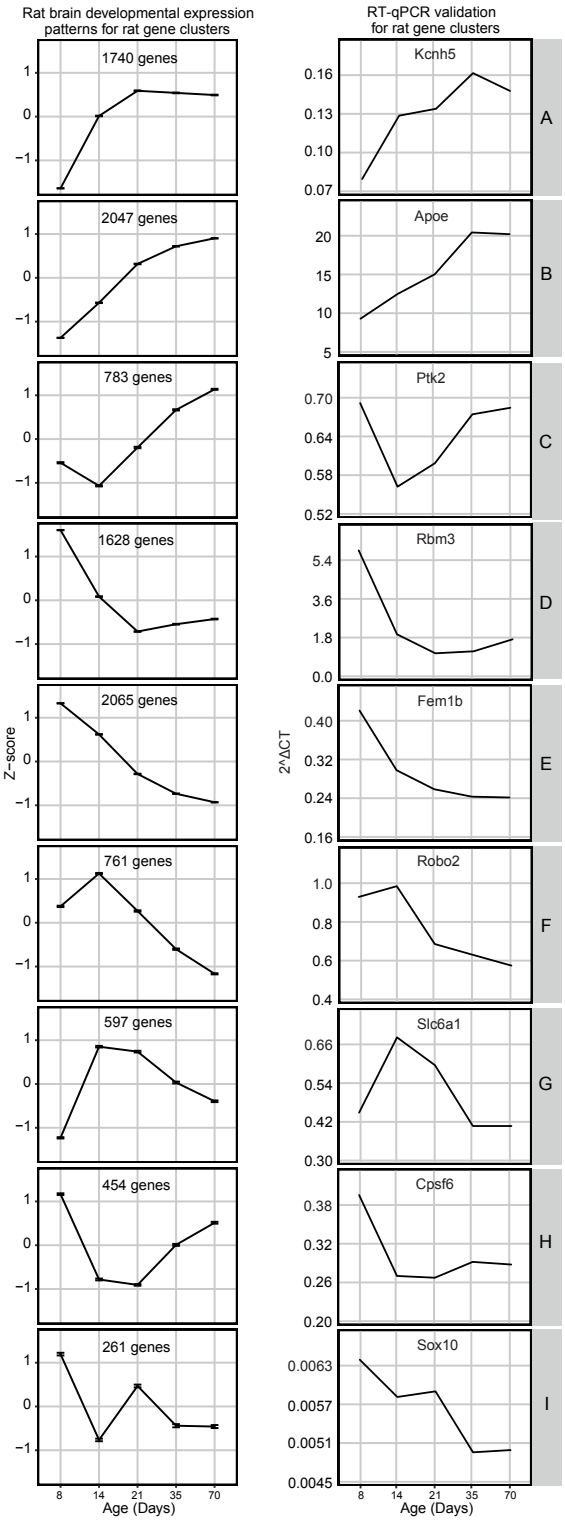
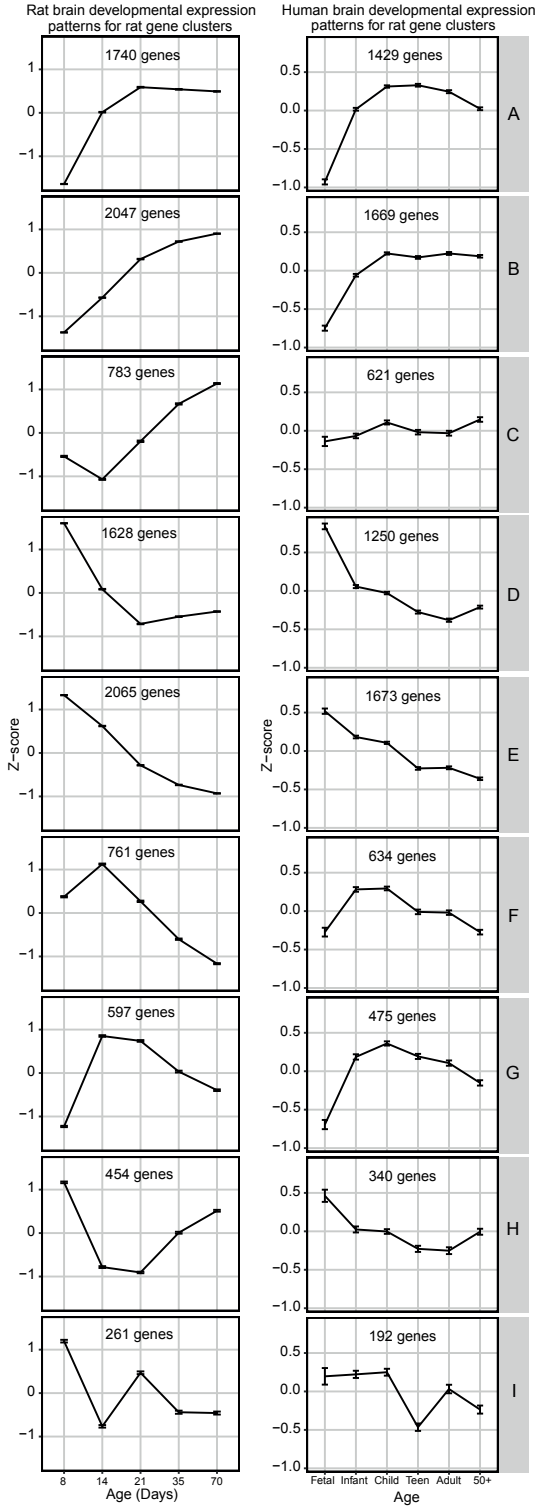
Supplementary material



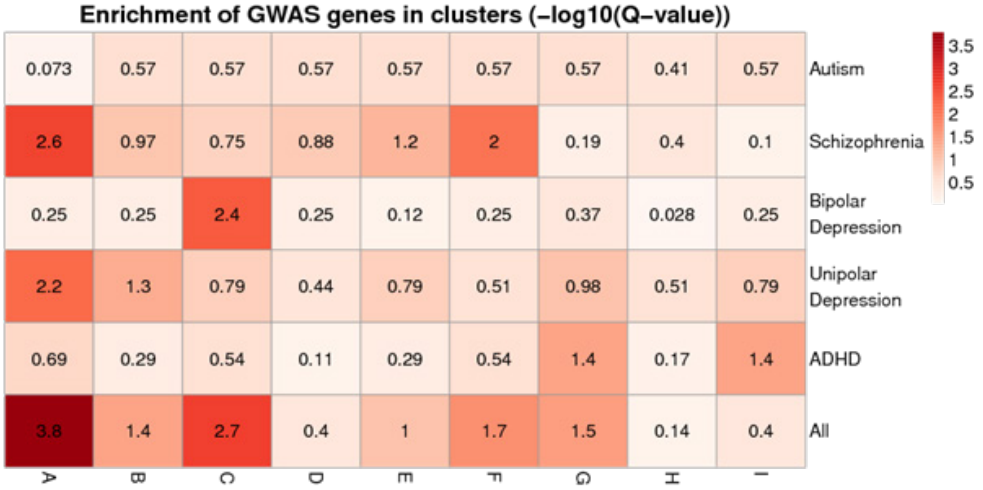
Supplementary Figure 1: Punch location of samples used for RNA-seq. (A) Position of the 8 consecutive slices in the adult brain (coronal slices adapted from Paxinos & Watson). Circle shows punch area. (B) Slices of developmental periods including the punch area (coronal 200 micron brain sections from Khazipov and colleagues[1]). Dashed line corresponds to horizontal midline which was used for determination of punching location in PND8 and PND14 slices, since the brain regions were harder to distinguish due to lower myelin levels. Prl = prelimbic cortex, Il = infralimbic cortex, Cg1 = cingulate cortex, area 1, fmi = forceps minor of the corpus callosum. CPu = caudate putamen.



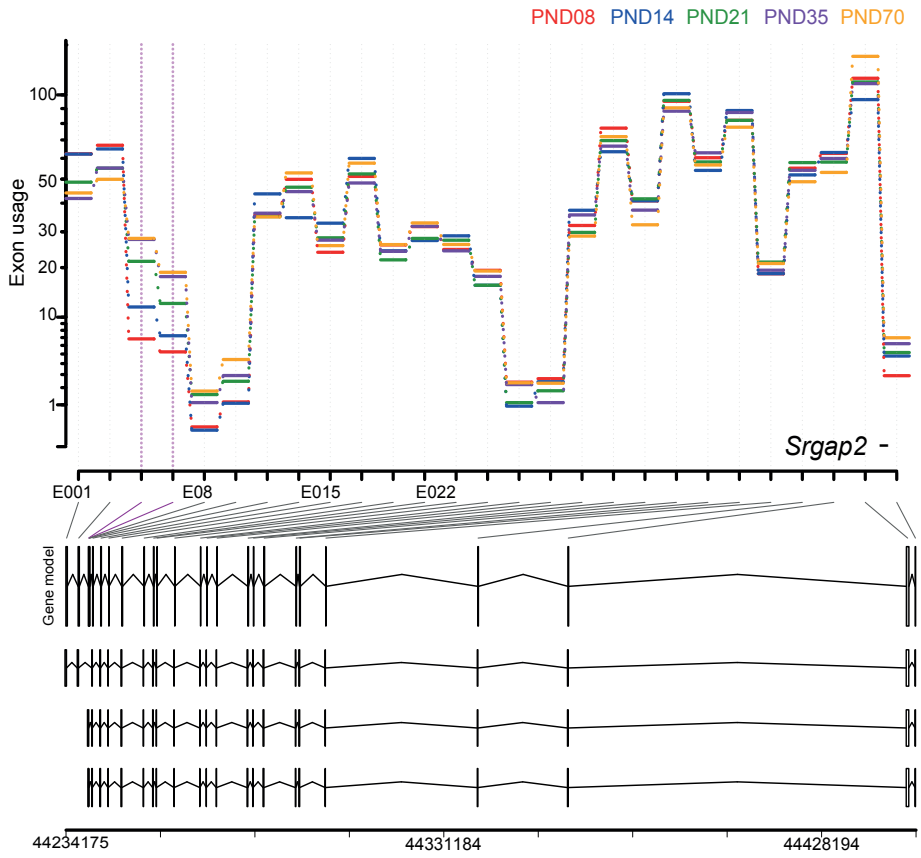
Supplementary Figure 2: Comparison of RNA-seq and RT-qPCR gene expression per cluster. Expression of RT-qPCR genes generally follows the same patterns as their corresponding RNA-seq clusters. Plots are shown for all clusters (A-I). Developmental time point is shown on the x-axis and z-score or 2^ΔCT is shown on the y-axis. Gene name or number of genes is shown above each graph. RT-qPCR gene expression is normalized by the average expression of two reference genes (Gabbr1, Kif5c). Error bars indicate the standard error of the mean.



Supplementary Figure 3: Comparison of human and rat brain developmental gene expression per cluster. Expression patterns per cluster generally follow the same pattern in humans (data from Jaffe *et al.*[2]) and rats. Plots are shown for all clusters (A-I). Developmental time point is shown on the x-axis and z-score is shown on the y-axis. Error bars indicate the standard error of the mean.



Supplementary Figure 4: **Enrichment of neuropsychiatric disorder GWAS genes per cluster**. Heatmap shows the -log10 transformation of the multiple testing-corrected p-value (q-value) for the enrichment of GWAS genes from each disorder (y-axis) in each cluster (x-axis). The hypergeometric distribution test was used to calculate the significance of the enrichment and multiple testing correction was done using the False Discovery Rate algorithm. The -log10 transformation facilitates visualization as it converts the exponential scale q-values to a linear scale (for instance $q=10^{-1}$ and $q=10^{-3}$ would correspond to 1 and 3 respectively after -log10 transformation).



Supplementary Figure 5: Alternative splicing of 3' exons in the medial prefrontal cortex of the rat. Graph shows expression per exon, corrected for overall expression level between time points. Colors represent the different time points during development in postnatal days (PND). Vertical purple dashed lines depict differentially expressed exons during development, caused by alternative exon usage. The upper x-axis depicts exon numbers. The y-axis shows normalized counts per exon after correcting for total transcript expression differences between the time points. The different isoforms are shown under the graph. The first one (gene model) represents the summary of all isoforms and the different isoforms are shown below. Differentially expressed exons are shown in purple. The lower x-axis (under the isoforms) shows the genomic location of the isoform. + or- behind the gene name represents the strand on which the gene is located. The *Srgap2* gene is an example showing alternatively spliced 3'exons (although first exon does not reach the threshold of significance). Exon E3 and E4 (genomically shorter splice variants) show higher expression in the later developmental stages compared to the early postnatal period.

Supplementary Table 1. Primers used for quantitative RT-PCR

Gene symbol	Ensembl number	Forward primer	Reverse primer
Kcnh5	ENSRNOG00000009542	CACATACCAGGAGCACAAGG	CAATGCCTTTGAAAATGTGG
Apoe	ENSRNOG00000018454	GTTCCTCCAGCTCCTTTTTG	CTGACCAGGTCCAGGAAGAG
Ptk2	ENSRNOG00000007916	GGCTGGTCATGACATACTGC	TGAGACCATTCCCATCCTTC
Rbm3	ENSRNOG00000005387	GACCAGGGATATGGAAGTGG	CCTGAGTAGCGGTCATAGCC
Fem1b	ENSRNOG00000007077	CAGTGGCACCATCAATGAC	CTCCACACCCCTCATCATC
Robo2	ENSRNOG00000029598	CGACATCAGTGATTTGCTCAG	GCAGGGGTTGGAGTAAAAAG
Slc6a1	ENSRNOG00000006527	AACACCACCAACATGACCAG	GGCTTGTCTAGCCCATCTG
Cpsf6	ENSRNOG00000005927	TCCCAAAGAATGAACTGCTTC	TTGGTGATGATGTGGGTAAAG
Sox10	ENSRNOG00000011305	TCTTTGGGGTGGTTGGAG	GCTGCTATCCAGGCTCACTAC
Gabbr1	ENSRNOG00000000774	TTTTCAGCCGCTTGGTTAG	ACATCACCACGGAGATTGTC
Kif5c	ENSRNOG00000004680	AAATTCAAAGGCGAGGAGAC	GCACAGGCATTGTAGACCTG

Supplementary Table 3. Number of differentially expressed genes

		PND8	PND14	PND21	PND35	PND70
PND8	**up**	-				
	down	-				
PND14	**up**	2638	-			
	down	2447	-			
PND21	**up**	3671	1677	-		
	down	3597	1564	-		
PND35	**up**	4038	2486	725	-	
	down	3889	2384	823	-	
PND70	**up**	4160	2809	1185	389	-
	down	4018	2711	1277	380	-

All other supplementary tables are available online

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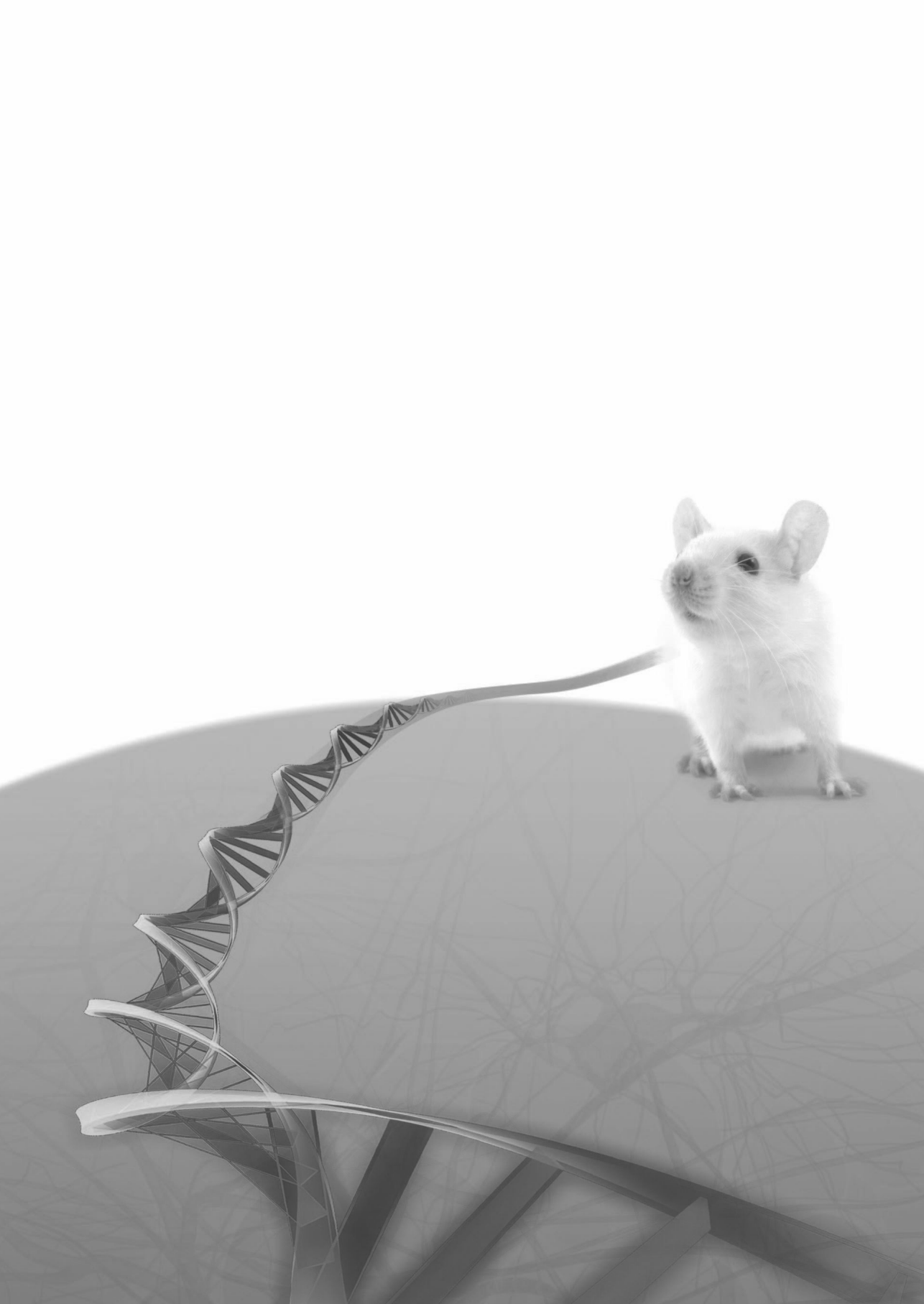

6

Genetic inactivation of the serotonin transporter dysregulates expression of neurotransmission and myelination genes in the postnatal medial prefrontal cortex of the rat

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In preparation

Abstract

Reduced expression of the serotonin transporter (5-hydroxytryptamine transporter, 5-HTT) in early-life has been associated with a delay in postnatal brain development and endophenotypes of a variety of neuropsychiatric and neurodevelopmental disorders in adolescence and adulthood. How a reduction in functional 5-HTT can disrupt neurodevelopment is still largely unknown. Here we studied genome-wide gene expression using transcriptome analysis (RNA-sequening, RNA-seq) and global levels of DNA (hydroxy)methylation (5(h)mC) using high-performance liquid chromatography-tandem mass spectrometry in 5-HTT wild-type (5-HTT$^{+/+}$) and 5-HTT homozygous knockout (5-HTT$^{-/-}$) rats across life (postnatal day (PND) 8, 14, 21, 35 and 70) in the medial prefrontal cortex (mPFC); a brain region with an extensive serotonergic innervation involved in several neuropsychiatric endophenotyes. We observed most gene expression changes in the mPFC during early postnatal life (PND8) and found at this time point an enrichment of genes linked to neuronal and developmental processes like neurotransmission, neuropeptide signaling and cell migration. Genome-wide 5(h)mC analysis showed a global increase in 5hmC in the mPFC during development in both genotypes and a significant increase in global 5hmC in 5-HTT$^{-/-}$ compared to 5-HTT$^{+/+}$ rats at PND35. The differences in the regulation of gene expression in 5-HTT$^{-/-}$ *versus* 5-HTT$^{+/+}$ rats during early postnatal life can dysregulate neurodevelopmental processes resulting in aberrant brain wiring and functioning. This might influence neurodevelopmental behavior and can result in lifelong consequences for executive functioning.

Introduction

Serotonin (5-hydroxytryptamine; 5-HT) is an important neurotransmitter in the central nervous system, where it has various functions including the regulation of mood[1], appetite[2], sleep[3,4] and learning and memory[5,6]. 5-HT is produced by serotonergic neurons in the raphe nuclei, which send their axons to regions throughout the brain. The serotonin transporter (5-HTT, encoded by *SLC6A4*) facilitates the reuptake of extracellular 5-HT in the presynaptic serotonergic neuron and thereby regulates extracellular 5-HT levels available for the activation of 5-HT receptors on the postsynaptic neuron. Besides its role as a neurotransmitter, 5-HT also acts as an important factor during neurodevelopment. 5-HT can promote neurodevelopmental processes, such as migration, neuronal outgrowth, and synaptogenesis[7-11]. In addition, while in the adult brain 5-HTT is only expressed in raphe neurons, 5-HTT expression is more widespread at early developmental stages. At mid-gestation (embryonic day 10.5 in mice[12]) expression of 5-HTT begins in the 5-HT neurons of the raphe nuclei, and subsequently 5-HTT expression emerges in the non-serotonergic neurons of the sensory system and corticolimbic pathway[13,14]. During the second postnatal week the 5-HTT expression in non-serotonergic neurons ends rapidly[14]. The functional implication of the transient 5-HTT expression is not completely clear. It has been proposed that it may serve to obtain the appropriate 5-HT levels important for developmental processes.

A common variation in the promoter region of *SLC6A4,* encoding 5-HTT, is the serotonin transporter promoter-linked polymorphic region (5-HTTLPR), which is associated with a variety of neuropsychiatric disorders. The short allelic variant (S-allele) of this variation is associated with reduced 5-HTT protein availability and function[15] compared with the long (L) form. S-allele carriers have an increased risk for developing depression[16], anxiety[17], substance abuse[18] and neurodegenerative disorders[19]. Because of the more widespread expression of 5-HTT in early-life and the developmental role of 5-HT, dysregulation of 5-HT in early-life might account for (additional) developmental problems. Indeed, the 5-HTTLPR has been shown to affect establishment of neuronal connectivity between brain regions, amongst others, the mPFC and the amygdala[20,21], possibly caused by disturbed 5-HT-dependent neurodevelopmental processes such as neurite outgrowth[8,11,22,23].

In order to study the effects of reduced 5-HTT expression in more detail, 5-HTT knockout (5-HTT$^{-/-}$) rats and mice have been developed[24,25]. These knockout rodents show a rather mild phenotype, i.e. they reach normal age (Homberg, unpublished findings), show normal coat conditions[26] and phenotype differences are not often readily apparent at first glance. However, 5-HTT$^{-/-}$ rodents show increased extracellular 5-HT levels[24], increased 5-HT innervation of the mPFC[8] and phenotype differences become apparent in several behavior tasks. These rodents display increased anxiety[27,28], depression-like behavior[28,29] and compulsive cocaine self-administration behavior at adulthood[18]. In addition, 5-HTT$^{-/-}$ rats show a delay in development compared to 5-HTT$^{+/+}$ rats, with the strongest effects observed in reflex development and motor-related behavior[30]. Furthermore, 5-HTT$^{-/-}$ mice show abnormally distributed interneurons[10] and altered neocortical cell density and layer thickness[8,31], caused

by disrupted migration of neurons during development[10]. The altered brain cytoarchitecture as a consequence of neurodevelopmental 5-HT dysregulation might be an important cause of the increased psychostimulant intake, anxiety and depression-like behavior observed in adult 5-HTT$^{-/-}$ rodents. In conclusion, human and rodent data show that a reduction in functional 5-HTT during development has (negative) consequences for early-life development and can enhance the risk of developing anxiety, depression and drug addiction in adulthood.

The prefrontal cortex (PFC), a brain region involved in the execution of cognitive and emotional functions[32-36], has an extensive serotonergic innervation[37,38] and has been implicated in neuropsychiatric disorders like anxiety, depression and drug addiction[39-41]. The PFC is one of the latest maturing brain regions, showing substantial changes during postnatal development[42,43]. From around birth till the second postnatal week 5-HTT is transiently expressed in non-serotonergic neurons in the PFC[14]. After the second postnatal week 5-HTT is only expressed in the serotonergic neurons from the raphe nucleus. That is, *Slc6a4* (encoding 5-HTT) transcription and translation takes place in raphe serotonergic cell bodies and 5-HTT protein is only present at the axon terminals which protrude to, amongst others, the PFC. In 5-HTT$^{-/-}$ rats 5-HTT protein is absent in the PFC (as well as all other non-raphe regions in the brain)[24] resulting in increased 5-HT innervation[8]. In explant cultures from developing 5-HTT$^{-/-}$ rat brains (embryonic day 16.5), 5-HT projections from the median raphe became strongly attracted by the medial PFC (mPFC) instead of being repelled by the mPFC, suggesting that 5-HT levels can influence connectivity in the mPFC[8]. In addition, the number of a class of callosal projection neurons was decreased in the developing 5-HTT$^{-/-}$ mPFC compared to 5-HTT$^{+/+}$ mPFC[8]. Furthermore, mPFC-regulated behavior, e.g. fear extinction[44] and cognitive flexibility[45,46], is disturbed in 5-HTT$^{-/-}$ rats. In humans, the 5-HTTLPR polymorphic region is associated with abnormalities in the mPFC. Children and adolescents carrying a homozygous S-allele (S/S) showed weaker connectivity in the superior mPFC compared to L-allele carriers[20]. The 5-HTTLPR is also associated with differences in mPFC-related behavior, like sustained attention[47], cognitive flexibility[48] and decision making[49], which might be a consequence of disturbed mPFC connectivity. However, to what extent dysregulation of the serotonergic system affects brain development in general and mPFC development in particular and whether disturbances during development predispose to the anxiety and depression-like behavior at adulthood is still unclear.

Neurodevelopment is a highly complex and strictly regulated process which is dependent on the precisely timed and coordinated expression of thousands of genes. Each specific neurodevelopmental process requires a different set of genes to be, spatially-defined, expressed at a specific time frame during development. There are several studies that investigated the influence of dysregulated 5-HT signaling on gene expression and they show altered expression of genes linked to the inflammatory system, the hypothamalus-pituitary-adrenal axis, neurotrophic factors, myelination and neurotransmitter systems[50-52]. However, most of these studies focussed on the effect in adulthood and studies on gene expression during neurodevelopment are limited. While several studies reported behavioral

and structural consequences of 5-HTT down-regulation during development, the molecular underpinnings contributing to these developmental changes are poorly understood. Studying genome-wide gene expression during development might reveal important new insights and might potentially lead to new targets for treatment of 5-HT-related disorders.

Gene expression can be regulated by epigenetic mechanisms, such as DNA (de) methylation, which is linked to gene regulation and developmental processes[53-57]. DNA methylation of cytosines at CpG sequences is catalyzed by a family of DNA methyltransferases (DNMTs), which show a spatiotemporal distribution during neurodevelopment. 5-hydroxymethylcytosine (5hmC), created by oxidation of 5-methylcytosine (5mC) by TET proteins, is thought to be an intermediate step in the active demethylation process[58]. However, more recent studies have indicated that 5hmC does not only serve as a DNA demethylation intermediate but also functions as a stable epigenetic mark[59,60]. Dysregulation of DNA methylation is seen in 5-HT-related neurodevelopmental and neuropsychiatric disorders, like autism spectrum disorder[56,61], schizophrenia[62], bipolar disorder[63], anxiety[64] and depression[65]. Whether the DNA methylation changes are a cause or consequence of these diseases and whether 5-HT dyregulation is involved in the methylation changes is unknown. Genome-wide gene expression and DNA (hydroxy)methylation changes during neurodevelopment as a consequence of reduced functional 5-HTT is a totally unexplored topic and might give important new insights.

To unravel the molecular mechanisms contributing to the structural and behavioral changes in the mPFC related to inherited 5-HTT deficiency, we studied genome-wide gene expression using transcriptome analysis (RNA-sequencing, RNA-seq) in the mPFC of 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ rats sacrificed at postnatal day (PND) 8, 14, 21, 35 and 70. We performed pair-wise comparisons and observed most differentially expressed genes during early postnatal life (PND8), with an enrichment for genes involved in neurotransmission and cell migration. Furthermore, several genes involved in myelination showed differential expression at multiple ages across development. In addition, we measured genome-wide DNA (hydroxy) methylation in mPFC tissue from 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ rats sacrificed at PND8, 14, 21, 35 and 70. We observed a significant increase in 5hmC levels in the 5-HTT$^{-/-}$ rats compared to the 5-HTT$^{+/+}$ rats at PND35. This, however, is not reflected in a higher number of differentially regulated genes between the two genotypes at this time point.

Materials and methods

Animals

5-HTT$^{-/-}$ rats (*Slc6a41Hubr*) were generated by ENU-induced mutagenesis[66]. Rats were housed in individually ventilated cages (40 x 35 x 23 cm, Greenline, Tecniplast, West Chester, USA) in temperature-controlled rooms (21 °C ± 1 °C) under standard 12-h light/dark cycle (lights on at 7:00 A.M.) with food (Sniff, long cut pellet, Bio Services, Uden, The Netherlands) and water available *ad libitum*. Pups were weaned at PND22. Rats were sacrificed by decapitation at five different time points; PND8, PND14, PND21, PND35 and PND70. For group 1, ten wild-type

(5-HTT$^{+/+}$) and ten 5-HTT$^{-/-}$ rats were sacrificed from three different nests per genotype per time point and were used for genome-wide expression analysis and Quantitative Reverse Transcription PCR (RT-qPCR) experiments. Half of the rats (n=5 rats/genotype/time point) were used for RNA-seq and RT-qPCR and the other half (n=5 rats/genotype/time point) only for RT-qPCR validation. For group 2 five rats were sacrificed per genotype per time point and used for genome-wide DNA (hydroxy)methylation analysis. Group 3 was used for validation of the results from group 2 and consisted of ten 5-HTT$^{-/-}$ and ten 5-HTT$^{+/+}$ rats sacrificed at PND35. All experiments were carried out according to the guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council 2003), the principles of laboratory animal care, as well as the Dutch law concerning animal welfare.

RNA extraction and double-stranded cDNA synthesis

Five samples per genotype per time point were used for RNA extraction. mPFC tissue was dissected from seven (PND8 and 14) or eight (PND21, 35 and 70) consecutive slices of 200 µm using a 2 mm punch needle and included the prelimbic and infralimbic cortex. For more details about the punching see Kroeze and colleagues[67]. Total RNA was isolated with QIAzol (RNeasy lipid tissue kit; QIAGEN, Venlo, The Netherlands) according to the manufacturer's recommendations. From each sample 2.5 µg RNA was used for rRNA depletion using the Ribo-Zero rRNA Removal Kit (Human/Mouse/Rat, Epicentre, Madison, Wisconsin, USA) according to the manufacturer's recommendations. RNA fragmentation reactions were performed using fragmentation buffer (5x; 200 mM Tris-Acetate, 500 mM potassium-Acetate, 150 mM magnesium-Acetate, pH 8.2) in a final concentration of 1x per reaction. Fragmentation reactions were incubated at 95°C for 4 min on a thermal cycler and placed on ice for 10 min. Fragmented rRNA-depleted RNA was purified using ethanol precipitation. First and second strand synthesis was performed as described by Kouwenhoven and colleagues[68].

Sequencing

DNA samples were prepared for sequencing by end repair of 5 ng total DNA as measured by Qubit dsDNA HS (Invitrogen). NEXTflex adaptors (Bioo Scientific, Austin, Texas, USA) were ligated to the DNA fragments, followed by post-ligation clean-up using Agencourt AMPure XP beads (Beckman Coulter, Woerden, The Netherlands), library amplification by PCR (10 cycles) and size selection (~300 bp) using Agencourt AMPure XP beads (Beckman Coulter). Quality control of DNA libraries prepared for sequencing was performed by qPCR and by running the products on a Bioanalyzer (Bio-Rad, Veenendaal, The Netherlands). Cluster generation and sequencing (50 bp, single end reads) were performed with the Illumina HiSeq 2000 sequencer according to standard Illumina protocols. Samples were sequenced to a depth of approximately 29 million reads per sample.

RNA-seq data processing

Reads were aligned to the rn4 rat genome assembly using the gsnap program[69], version

2012-07-20. Transcript quantification analysis was done with Cufflinks[70] using the rat Ensembl transcriptome (version 69). Cufflinks quantifies gene expression as FPKM (fragments per kilobase per million mapped reads), which corrects for different transcript lengths and sample sequencing depths. Short genes (5201 genes shorter than 200 base pairs, including small nuclear RNAs, small nucleolar RNAs, microRNAs and miscellaneous RNAs) were removed. This is because FPKMs of short genes tend to be more affected by background noise, since the correction for gene length gives more weight to the reads on short genes, thereby possibly increasing the FPKM to biologically irrelevant levels. To check for possible mistakes and errors during the sequencing of the RNA, the reads were assessed for the quality of calling the nucleotides in the reads quantified by Phred scores and the enrichment of not-called bases at specific locations in the reads[71]. The FPKMs calculated by Cufflinks were used to perform a principal component analysis (PCA) to look at variance between the samples[72]. PCA was performed on log10 transformed FPKM values.

Differential expression analysis, clustering and gene function

Differential expression analysis was performed using the CuffDiff program[73] version 2.2.1, using the Ensembl database[74] version 69 rat transcriptome annotation. The expression values from Cufflinks were further normalized by CuffDiff across the samples in the experiment by scaling them according to the geometric mean of the samples. Based on these expression data, CuffDiff calculates differentially expressed genes ($p<0.01$, FPKM>1, fold change>1.2) between the 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats for all five time points, taking replicates per genotype into account. The differentially expressed genes were clustered based on the standardized log10 transformed FPKMs (Z-scores) with hierarchical clustering using Multiexperiment Viewer[75]. The Pearson correlation coefficient was used as a measurement of distance in the hierarchical clustering. Gene ontology (GO) analysis was performed using the DAVID website[76]. DAVID calculates modified Fisher Exact p-values, EASE Scores, for each gene category[77]. Ingenuity Pathway Analysis software package (Ingenuity systems, www.ingenuity.com) was used for pathway analysis and analysis of Top Diseases and Bio Functions. Ingenuity calculates *p*-values for the enrichment of each gene category using the right-tailed Fisher exact test, taking into consideration both the total number of molecules from the analyzed data set and the total number of molecules linked to the same gene category according to the Ingenuity Knowledge Base. We generated a molecular network by integrating the results of the bioinformatics analyses from PND8 with systematic literature searches. For all genes linked to one of the five most significantly enriched GO-terms and genes linked to one of the five most significantly enriched Ingenuity pathways (except Hepatic Fibrosis / Hepatic Stellate Cell Activation), we looked at the available information in the NCBI Gene database and subsequently we searched PubMed using the search terms 'prefrontal cortex', 'neurodevelopment', 'signal transduction', 'serotonin', 'gutamate', in combination with the name of each candidate gene or their protein name. Guided by the literature we found, we also searched PubMed for functional interactions between the candidate genes/encoded

proteins.

Quantitative Reverse Transcription PCR (RT-qPCR)

RNA-seq validation was performed by RT-qPCR analysis of ten selected genes. Primers were designed using Primer3 online software (http://frodo.wi.mit.edu). See Supplementary Table 1 for primer sequences. Complementary DNA (cDNA) was synthesized using 500 ng of total RNA in a reverse transcription reaction using iScript cDNA Synthesis Kit according to manufacturer's protocol (Bio-Rad, Veenendaal, The Netherlands). qPCR reactions were performed in a 7500 Fast Real Time PCR System (Applied Biosystems, Foster City, CA, USA) using the SYBR Green fluorescence quantification system (GoTaq® qPCR Master Mix, Promega Benelux BV, Leiden, The Netherlands). Thermal cycling was initiated with incubation at 95°C for 10 min followed by 40 cycles of 95°C for 30 sec and 60°C for 1 min. To normalize the cDNA content of the samples, we used the comparative threshold cycle (CT) method[78], which consists of the normalization of the number of target gene copies versus an endogenous reference gene *Gabbr1* (stable gene in the RNA-seq dataset throughout all ages based on the Coefficient of Variation (CV = stddev/mean).

Genome wide DNA (hydroxy)methylation

DNA of mPFC tissue was isolated using the ReliaPrep gDNA Tissue miniprep system (Promega, Leiden, The Netherlands) according to the manufacturer's recommendations. 200 ng of DNA was degraded into individual nucleosides using DNA degradase plus (Zymo Research, Irvine, CA). 5mC and 5hmC levels were measured as described by Kroeze *et al*[58]. In short, the individual nucleosides (dG, mdC and hmdC) were measured using a high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) system (Waters, Milford, MA, USA). Calibration standards containing internal standard solutions were measured to obtain area-based linear regression curves for quantification. The 5mC and 5hmC levels were calculated as a concentration percentage ratio of % 5-methyl-2′-deoxycytidine/2′-deoxyguanosine (%mdC/dG) and % 5-hydroxymethyl-2′-deoxycytidine/2′-deoxyguanosine (%hmdC/dG), respectively.

Statistical analysis

Statistical analysis of RT-qPCR and 5(h)mC data was carried out using the IBM Corporation Statistical Package for the Social Sciences (SPSS) version 20.0 (IBM Corp, Armonk, NY, USA). Data were analyzed using independent samples *t* tests (corrected p-value was used when equal variance was not assumed). Outliers (data points further than 3 interquartile ranges from the nearer edge of the box plot) were excluded from the analysis. Independent samples *t* tests were performed two-sided and the level of statistical significance was set at $p<0.05$.

Results

Validation of the knockout model and general differences between PFC samples

RNA-seq was performed and the reads were aligned to the rn4 rat genome assembly. To validate our knockout model, reads spanning the third exon of the *Slc6a4* gene, corresponding to chromosome 10 position 67152790 to 67153009 from the Brown Norway rat genome (rn4 assembly[79]), were extracted and examined for the described mutation by calling differences between the extracted reads and the reference genome[80]. In the 5-HTT$^{-/-}$ rats this third exon should contain a C to A transversion at nucleotide 3924 (c.3924C>A, based on ENSRNOG0000003476), resulting in a premature stop codon (p.C1308X)[24]. All 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ replicates were pooled because the coverage of the 5-HTT gene by individual samples was too low. The third exon was more extensively covered in the 5-HTT$^{+/+}$ samples (23 reads) compared to the 5-HTT$^{-/-}$ samples (9 reads). Indeed, the 5-HTT$^{-/-}$ rat differed from the 5-HTT$^{+/+}$ rat and the reference at nucleotide position 3924, matching the described mutation by Homberg and colleagues[24]. It should be noted that this variant was based on only a single read that covers this genomic region (Figure 1A). In addition, the 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ sequences both differed from the reference at nucleotide position 3891, indicating a single nucleotide polymorphism (rs8154473, encoding for the same amino acid) between the reference (Brown Norway rat) and our Wistar rats (Figure 1A, deviations highlighted in orange).

Differential expression analysis with CuffDiff[70] was performed to obtain the normalized gene expression values (FPKM values). A PCA was performed on log10 transformed FPKM values to examine variations between the samples. Principal component 1 (PC1) (19.2%) seems to separate samples according to age, with the PND8 samples on the left side and the PND70 samples on the right side (Figure 1B). PC2 (6.7%) separated 4 samples from the rest. Because these 4 samples were from different time points and from both 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ rats these might be potential outliers and therefore we decided to exclude them for further analyses. Samples of 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ rats are not separated in this analysis, indicating small gene expression differences in mPFCs between 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ rats.

Differential expression analysis and clustering

For each time point differentially expressed genes ($p<0.01$, FPKM>1, fold change>1.2) between 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats were determined using five samples per genotype. The highest number of differentially expressed genes between 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats was found at PND8 (615 genes). The other time points showed lower numbers of differentially expressed genes, namely 135 genes for PND14, 126 genes for PND21, 97 genes for PND35 and 115 genes for PND70 (Figure 2A, Online Supplementary Table 2). The *Slc6a4* gene (encoding for 5-HTT) showed differential expression only at PND8 (Figure 2B). 5-HTT is hardly expressed at the other time points. As a consequence no significant differences were observed at these time points. Of the differentially expressed genes, 123 genes were differentially expressed at more than one time point. None of these genes showed differential expression at all five

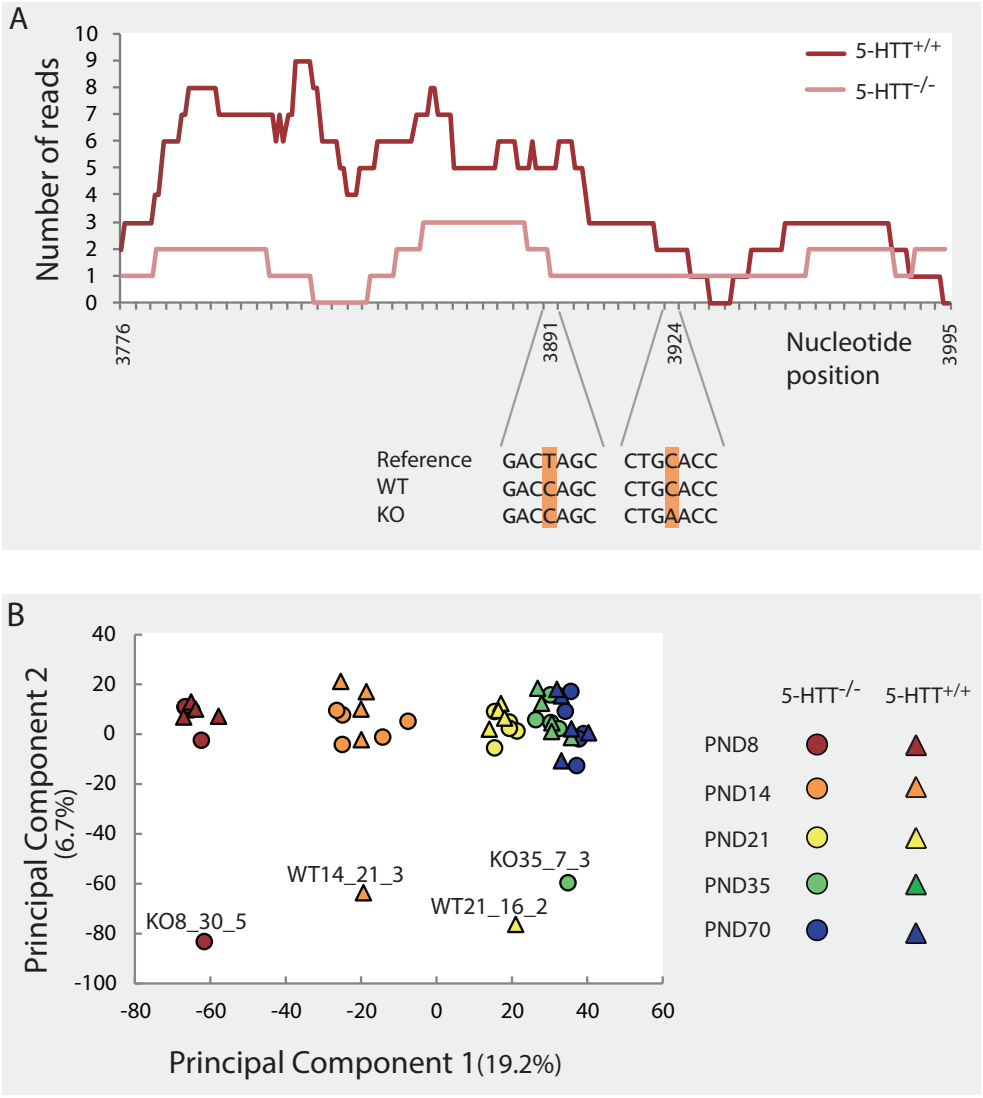

Figure 1. Comparison of 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ samples to the reference genome and principal component analysis. A: Comparison of reads from 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ samples to the reference *Slc6a4* gene. The number of reads that span a position in the third exon of the *Slc6a4* gene is depicted on the y-axis in the graph. The x-axis depicts the nucleotide position based on the ensembl gene ENSRNOG00000003476. 5-HTT$^{+/+}$ is indicated by the red line and 5-HTT$^{-/-}$ is indicated by the pink line. Sequences deviating from ENSRNOG00000003476 (based on the Brown Norway rat, rn4 assembly[79]) are shown under the graph. The highlighted nucleotides correspond to the locations in the graph. B: Principle component analysis (PCA). PCA was performed on log10 transformed Fragments Per Kilobase per Million mapped reads (FPKM) values (a pseudocount of 0.01 was added to avoid infinite values) and small genes were excluded. The axes are labelled by the principal component they represent and the percentage of explained variance by the principal component. Circles represent 5-HTT$^{-/-}$ samples and triangles represent 5-HTT$^{+/+}$ samples. Red represents PND8, orange represents PND14, yellow represents PND21, green represents PND35 and blue represents PND70. Explanation sample numbers: WT (wild-type) = 5-HTT$^{+/+}$, KO (knockout) = 5-HTT$^{-/-}$. Genotype is followed by postnatal day, litter number and number of the rat in the litter.

time points (Supplementary Online Table 2). Manual checking for genes with overlapping function revealed four genes involved in myelination (*Cldn11, Plp1, Mag, Mbp*[81]), which is a process that has been associated with 5-HT signaling before[51,82,83]. All four genes showed differential expression ($p<0.01$) between 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats at more than one time point and all showed a similar expression pattern (Figure 2C-F). Although the difference in expression of these genes between 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats did not reach significance at every specific time point, in general we observed a lower expression of myelin-related genes in 5-HTT$^{-/-}$ rats compared to the 5-HTT$^{+/+}$ rats at PND8, 21 and 70 and a higher expression in 5-HTT$^{-/-}$ compared to 5-HTT$^{+/+}$ rats at PND14 (Figure 2C-F).

The differentially expressed genes (all time points combined) were clustered with hierarchical clustering based on Z-scores (Figure 3). The 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ cluster together per age, indicating that in the group of differentially expressed genes between the 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ mPFC samples, the differences in time were larger than the differences between genotypes. In addition, gene expression patterns seem to differ most between early (PND8 and 14) and later ages (PND21, 35 and 70). These results are consistent with the data from the PCA (Figure 1).

In conclusion, most differences in gene expression in the mPFC were observed at the earliest time point measured in this study, PND8. These expression differences might affect developmental processes which can result in lifelong consequences for brain functioning.

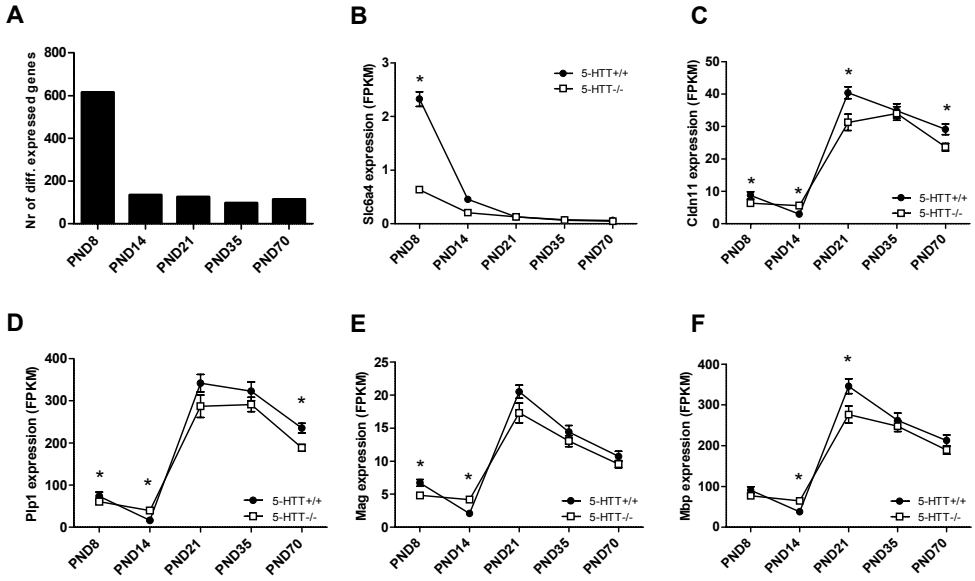

Figure 2. Differential expression analysis in prefrontal cortex RNA of 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats. (A) The number of differentially expressed genes per time point. (B-F) Graphs of expression patterns of the serotonin transporter gene (*Slc6a4*) and four myelin-related genes. Fragments Per Kilobase per Million mapped reads (FPKM) at five developmental time points in 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ medial prefrontal cortex are shown. (B) *Slc6a4*, (C) *Cldn11,* (D) *Plp1*, (E) *Mag*, (F) *Mbp*. *p<0.01 based on RNA-seq statistical analysis.

Gene ontology and pathway analysis

To functionally categorize the differentially expressed genes, GO analysis was performed. The most significantly enriched GO-terms for the different time points were **transmission of nerve impulse** (PND8), **gas transport** (PND14), **response to organic substance** (PND21), **translational elongation** (PND35) and **response to nicotine** (PND70). See Table 1 for the top five GO-terms per time point. For a complete list of GO terms, see Online Supplementary Table 2. In addition, we used Ingenuity which is a tool that models, analyzes, and understands complex biological systems by linking incoming data with known information regarding molecular interactions, cellular phenotypes, and disease processes. Ingenuity core analysis was used to look for significantly enriched pathways. The most significantly enriched pathways for the different time points were **G protein coupled receptor signaling** (PND8), **neuroprotective role of THOP1 in alzheimer's disease** (PND14), **chondroitin sulfate degradation** (Metazoa) (PND21), **EIF2 signaling** (PND35) and **growth hormone signaling** (PND70) (Table 2). Since at PND14, 21, 35 and 70 a relative low number of differentially expressed genes (n<135) were found, the pathway analysis showed higher p-values and was therefore less reliable for these time points compared to PND8.

As we observed most expression changes between 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats at PND8 and GO and pathway analysis showed most reliable results in subsequent analysis, we focused on gene expression data of PND8. For PND8, three of the top five most significantly enriched GO-terms using DAVID were involved in neurotransmission (**transmission of nerve impulse**, **synaptic transmission** and **cell-cell signaling**), with 24 genes that were linked to all three GO-terms (Supplementary Table 3). In addition, **neuropeptide signaling pathway** is in the top five GO-terms, which contains peptides involved in a variety of neurodevelopmental processes like cell adhesion (e.g. *Gpr56*) and cell migration/axon guidance (*e.g. Sstr2*). Furthermore, **cell migration**, a process important in brain development, is in the top five GO-terms and includes several factors and receptors involved in cell migration and axon guidance (e.g. *Ntn1, Nrp2, Dcc, Sema3c*). Three of the top five Ingenuity pathways for PND8 were involved in signal transduction; that is **G protein coupled receptor signaling**, **cAMP-mediated signaling** and **Gi signaling**. The **G protein coupled receptor signaling** pathway included several serotonergic receptors (*Htr4, Htr7, Htr2a* and *Htr2c*) and also showed substantial overlap with the **cAMP-mediated signaling pathway** (15 genes) and the **Gi signaling pathway** (9 genes) (Supplementary Table 4).

Based on the results of DAVID (genes linked to neurotransmission), Ingenuity (genes linked to signal transduction) and systematic literature search we built a potential network involving 5-HT and glutamate signaling in the mPFC (Figure 4). Among the receptors showing differential expression at PND8 is the 5-HT receptor gene *Htr2a,* which upon activation can stimulate glutamate release[84]. Several genes encoding for glutamatergic receptors (*Grm2, Grm4, Gria4, Grin2a)* were also differentially expressed in the mPFC. In addition, certain genes involved in synthesis (*Ddc*), vesicular transport (*Slc17a7*[85]*, Sv2b*[86]*, Dynll1*[87]*, Syn2*[88]) and release (*Vamp2*[89]*, Cplx3*[90]) of neurotransmitters and genes involved in downstream signal

transduction pathways (*Rgs4*[91], *Gng3*[92], *Prkcb*[93]) were downregulated in the 5-HTT$^{-/-}$ mPFC, suggesting an overall decrease in neurotransmission. Glutamic acid decarboxylases, encoded by *Gad1* and *Gad2*[94], catalyzing the synthesis of gamma-aminobutyric acid (GABA) from glutamate, were upregulated in the 5-HTT$^{-/-}$ mPFC.

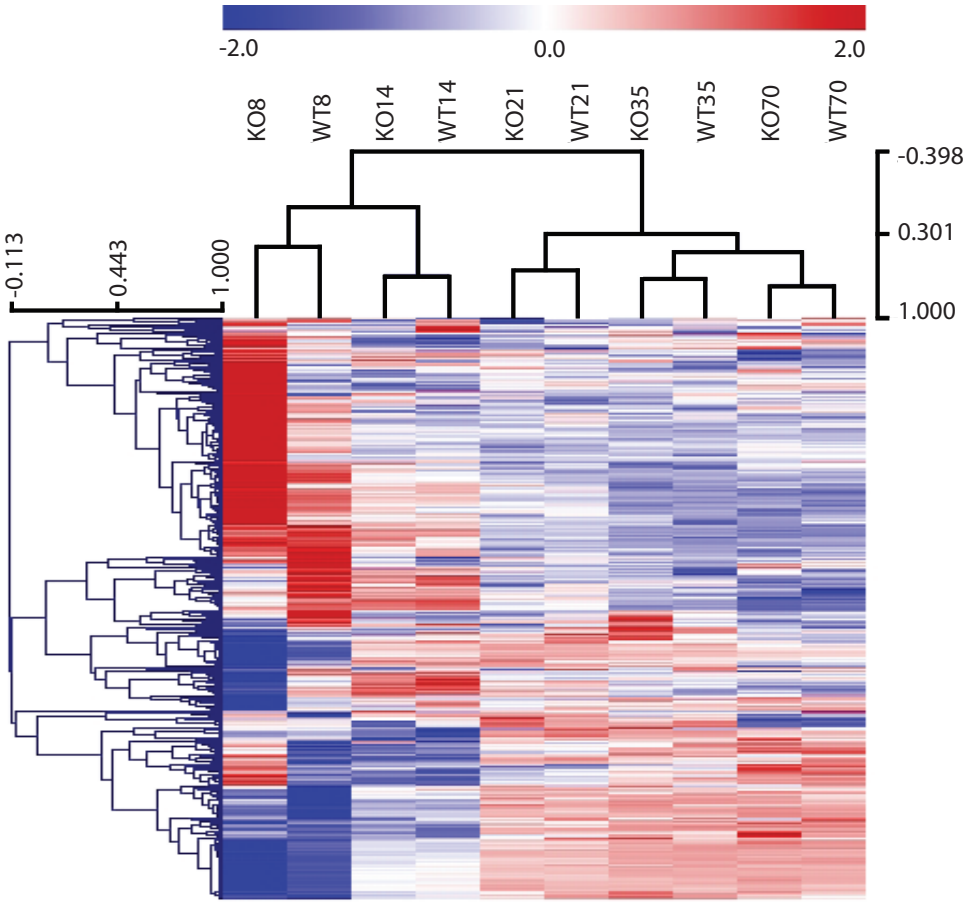

Figure 3. Hierarchical clustering of the differentially expressed genes between 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ mPFC samples. The genes are log10 transformed (a pseudocount of 0.01 was added to avoid infinite values), standardized per gene (Z-scores) and clustered with the Pearson correlation factor as a measure of distance. Red indicates above average expression and blue indicates below average expression.

Table 1. Significantly enriched GO-terms (biological process) affected by 5-HTT$^{-/-}$ in the medial prefrontal cortex

	GO-term	Nr of genes	Example genes	p-value
PND8	Transmission of nerve impulse	29	*Slc6a4, Svb2, Vamp2, Grm2*	3.63E-08
	Neuropeptide signaling pathway	14	*Cartpt, Nmur2, Sstr2, Pdyn*	1.77E-07
	Synaptic transmission	24	*Slc6a4, Svb2, Vamp2, Grm2*	2.13E-07
	Cell-cell signaling	30	*Slc6a4, Svb2, Vamp2, Grm2*	2.70E-07
	Cell migration	26	*Gja1, Smo, Vamp2, Met*	3.21E-07
PND14	Gas transport	5	*Hba-a2, Hbb, Aqp1, Loc689064*	6.16E-06
	Oxygen transport	4	*Hba-a2, Hbb, Loc360504, Loc689064*	6.80E-05
	Regulation of acute inflammatory response	4	*Npy5r, Anxa1, Cd46, Cr1l*	1.26E-03
	Cell adhesion	11	*Cntnap5b, Mag, Cldn11, Ctgf*	1.34E-03
	Biological adhesion	11	*Cntnap5b, Mag, Cldn11, Ctgf*	1.34E-03
PND21	Response to organic substance	18	*P2rx4, Gng7, Igf2, Fos*	3.37E-05
	Chemical homeostasis	13	*Cartpt, Vgf, Egr2, Cldn11*	3.96E-05
	Regulation of action potential in neuron	6	*Cldn11 Mbp, Pllp, Mal*	5.38E-05
	Response to extracellular stimulus	10	*Cartpt, Igf2, Fos, A2m*	8.92E-05
	Homeostatic process	15	*Cartpt, Vgf, Egr2, Cldn11*	9.20E-05
PND35	Translational elongation	5	*Rpl10, Rpl19, Rps16, Rps7*	1.03E-03
	Regulation of blood pressure	4	*Cyp11b1, Ptgs2, Calca, Ephx2*	1.36E-02
	Translation	7	*Rpl27a, rpl10, Rpl19, Rps16*	1.50E-02
	Response to glucocorticoid stimulus	4	*Fos, Ptgs2, Dusp1, Plat*	2.06E-02
	Response to corticosteroid stimulus	4	*Fos, Ptgs2, Dusp1, Plat*	2.38E-02
PND70	Response to nicotine	3	*Igf2, Htr2c, Chrna5*	1.01E-02
	Fatty acid metabolic process	5	*Crem, Acsm3, Plp1, Decr1*	1.93E-02
	Cell adhesion	7	*Pcdhga1, Hapln4, Nlgn2, Cldn11*	4.01E-02
	Biological adhesion	7	*Pcdhga1, Hapln4, Nlgn2, Cldn11*	4.01E-02
	Behavioral response to nicotine	2	*Htr2c, Chrna5*	4.29E-02

Table 2. Top canonical pathways affected by 5-HTT$^{-/-}$ in the medial prefrontal cortex

	Pathway	Overlap	Example genes	p-value
PND8	G-Protein Coupled Receptor Signaling	8.0 % 20/251	*Grm2, Grm4, Htr4, Htr2a, Rgs4*	2.76E-06
	Hepatic Fibrosis / Hepatic Stellate Cell Activation	8.7 % 16/183	*A2m, Col19a1, Col1a2, Igf2, Igfbp3*	8.52E-06
	cAMP-mediated signaling	7.9 % 17/215	*Grm2, Grm4, Htr4, Htr7, Rgs4*	1.69E-05
	Serotonin Receptor Signaling	17.1 % 7/41	*Slc6a4, Htr4, Htr7, Htr2a,Htr2c*	4.67E-05
	Gi Signaling	8.6 % 10/116	*Grm2, Grm4, Gng3, Rgs4, Sos2*	4.63E-04
PND14	Neuroprotective Role of THOP1 in Alzheimer's Disease	7.5 % 3/40	*Nts, Pdyn, Prkag1*	9.03E-04
	IGF-1 Signaling	4.2 % 4/96	*Ctgf, Igfbp2, Igfbp4, Prkag1*	1.14E-03
	EIF2 Signaling	2.2 % 4/183	*Rpl19, Rps2, Rps26, Rps29*	1.13E-02
	mTOR Signaling	2.1 % 4/187	*Prkag1, Rps2, Rps26, Rps29*	1.22E-02
	Notch Signaling	5.3 % 2/38	*Hes5, Mag*	1.40E-02
PND21	Chondroitin Sulfate Degradation (Metazoa)	13.3 % 2/15	*Cd44, Hyal1*	2.34E-03
	Dermatan Sulfate Degradation (Metazoa)	12.5 % 2/16	*Cd44, Hyal1*	2.67E-03
	Growth Hormone Signaling	4.3 % 3/69	*A2m, Fos, Igf2*	4.59E-03
	Tight Junction Signaling	2.4 % 4/167	*Cldn11, Fos, Myh11, Pvrl1*	8.90E-03
	RhoGDI Signaling	2.3 % 4/171	*Cd44, Cdh7, Gng7, Rhog*	9.65E-03
PND35	EIF2 Signaling	3.3 % 6/183	*Rpl19, Rpl41, Rps16, Rpl27a, Rps7*	2.94E-05
	cAMP-mediated signaling	2.3 % 5/215	*Aplnr, Dusp1, Dusp4, Pde10a, Pde7b*	6.86E-04
	G-Protein Coupled Receptor Signaling	2.0 % 5/251	*Aplnr, Dusp1, Dusp4, Pde10a, Pde7b*	1.37E-03
	IL-8 Signaling	2.2 % 4/182	*Fos, Ptgs2, Rhoj, Vegfb*	2.97E-03
	ILK Signaling	2.2 % 4/186	*Fos, Ptgs2, Rhoj, Vegfb*	3.21E-03
PND70	Growth Hormone Signaling	4.3 % 3/69	*A2m, Fos, Igf2*	3.97E-03
	EIF2 Signaling	2.2 % 4/183	*Rpl17, Rpl19, Rpl30, Rps2*	1.02E-02
	Antigen Presentation Pathway	5.6 % 2/36	*Psmb8, Tap2*	1.19E-02
	Methionine Salvage II (Mammalian)	33.3 % 1/3	*Bhmt*	1.37E-02
	Role of Tissue Factor in Cancer	2.7 % 3/110	*Cyr61, Egr1, Hck*	1.43E-02

Abbreviations: cAMP: cyclic adenosine monophosphate, EIF2: eukaryotic initiation factor 2, IGF-1: insuline-like growth factor 1, ILK: Integrin-linked kinase, IL-8: Interleukin 8, mTOR: mechanistic target of rapamycin, RhoGDI: Rho GDP-dissociation inhibitor

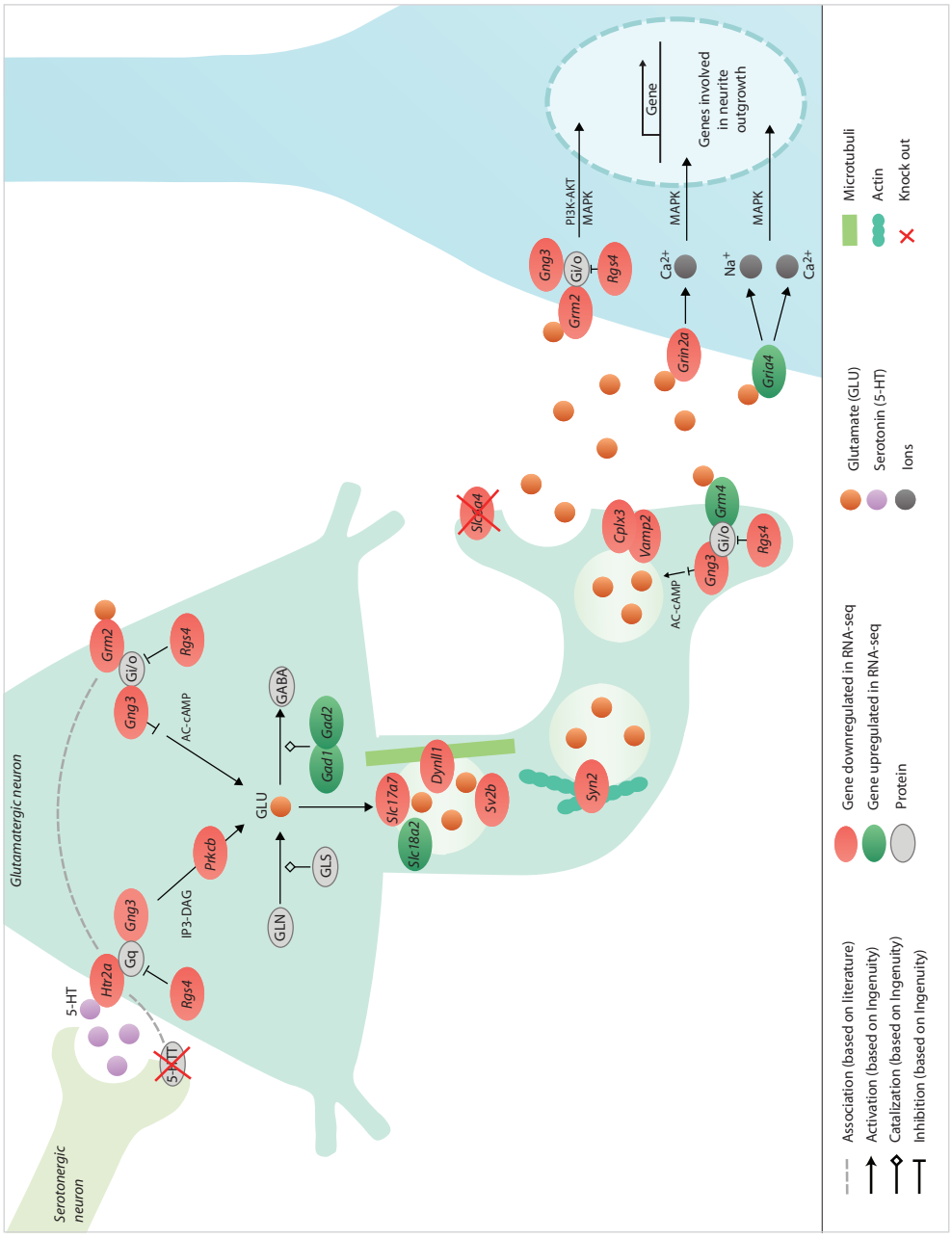
Glutamatergic neuron
Serotonergic neuron
5-HT
5-HTT
Htr2a
Gq
Gng3
Rgs4
IP3-DAG
Prkcb
Grm2
Gi/o
Gng3
Rgs4
AC-cAMP
GLN
GLS
GLU
GABA
Gad1
Gad2
Slc17a7
Slc18a2
Dynll1
Syt2b
Syn2
Slc6a4
Cplx3
Vamp2
Gng3
Gi/o
Grm4
Rgs4
AC-cAMP
Grin2a
Gria4
Ca2+
Na+
Ca2+
MAPK
MAPK
Genes involved in neurite outgrowth
Gene
PI3K-AKT
MAPK
Gng3
Gi/o
Grm2
Rgs4
Association (based on literature)
Activation (based on Ingenuity)
Catalization (based on Ingenuity)
Inhibition (based on Ingenuity)
Gene downregulated in RNA-seq
Gene upregulated in RNA-seq
Protein
Glutamate (GLU)
Serotonin (5-HT)
Ions
Microtubuli
Actin
Knock out

Figure 4. Potential neurotransmitter pathway affected by genetic 5-HTT inactivation at PND8. Serotonin (5-HT) is produced in the raphe nucleus and transported in vesicles to the axon terminals located in, amongst others, the prefrontal cortex (PFC). 5-HT can be released from these vesicles into the synaptic space, where it can activate subtypes of receptors. 5-HT_{2A}, encoded by the *Htr2a* gene, is a Gq coupled 5-HT receptor present on glutamatergic pyramidal cells in deep cortical layers (V and VI)[84]. Heterotrimeric G protein complexes are made up of alpha, beta and gamma (such as *Gng3*) subunits. RGS proteins, such as *Rgs4*, are able to deactivate G protein subunits[91]. The most prominent pathway stimulated by 5-HT_{2A} is the diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3) pathway (IP3-DAG)[93] which activates Protein kinase C (encoded by *Prkcb*). Studies have shown that 5-HT_{2A} receptor signaling is reduced in (adult) 5-$HTT^{-/-}$ mice (grey dotted line)[95,96]. Upon activation, 5-HT_{2A} can trigger glutamate (GLU) release from the pyramidal cell[84]. GLU is synthesized by the conversion of glutamine (GLN) catalyzed by glutaminase (GLS) and packaged into synaptic vesicles by the vesicular glutamate transporters (VGLUTs; encoded by, amongst others, *Slc17a7*)[85]. Glutamic acid decarboxylases, encoded by *Gad1* and *Gad2*, catalyze the synthesis of gamma-aminobutyric acid (GABA) from GLU[94]. Long distance transport of synaptic vesicles, containing synaptic vesicular proteins (e.g. synaptic vesicle glycoprotein 2B encoded by *Sv2b*[86]), occurs by kinesin and dynein (encoded by, amongst others, *Dynll1*) in the microtubule-rich axon and vesicles then travel through the actin-rich cortex at the nerve terminal on a myosin motor[87]. Synapsins (encoded by, amongst others, *Syn2*) regulate the release of neurotransmitters by preventing vesicles from migrating to the presynaptic membrane by binding synaptic vesicles to actin[88]. The SNARE complex, composed of three membrane-associated proteins (encoded by, amongst others, *Vamp2*), mediate the fusion of vesicles with the presynaptic membrane[89]. Complexin (encoded by, amongst others, *Cplx3*) acts as a positive regulator of synaptic vesicle exocytosis, and binds selectively to the neuronal SNARE complex[90]. Extracellular GLU can activate a variety of receptors. Each receptor can activate its own signal transduction pathway inside the postsynaptic neuron. mGlu2 receptors, encoded by the *Grm2* gene, are localized in the pre- and postsynaptic membrane[97-99]. Activation of presynaptic mGlu2 receptors negatively modulates the release of GLU by providing a feedback that prevents excessive GLU release[100,101] and regulates the release of other neurotransmitters[102]. Postsynaptic mGlu2 receptors can regulate neuronal excitability via the modulation of ion channels[100]. mGlu2 is a Gi/o coupled receptor which inhibits the adenylate cyclase (AC) – Cyclic adenosine monophosphate (cAMP) pathway[99] (pre-synaptic mGlu2) and can also activate the MAPK and PI3K-AKT pathways (post-synaptic mGlu2)[93,103]. Studies have shown that mGlu2 can form a heterocomplex with the 5-HT_{2A} receptor[97,104] (grey dotted line). mGlu4, encoded by *Grm4*, is an autoreceptor localized at the active zone of boutons which inhibits the AC – cAMP pathway[105,106]. AMPA receptor (encoded by, amongst others, *Gria4*) activation can result in sodium and calcium influx. The intracellular signaling pathways used by AMPA receptors are not fully understood. One potential pathway is PI3K-dependent activation of MAPK[107]. NMDA receptor (encoded by, amongst others, *Grin2a*) activation results in an increase in calcium influx, which in turn activates MAPK signaling-related cascades[108]. Glutamate receptor signaling might affect transcription of genes involved in synapse formation and neurite outgrowth. For example, NMDA receptor signaling can influence netrin 1 (*Ntn1*) gene expression, which is a diffusible protein involved in cell migration and axon guidance and linked to 5-HT[109,110]. In addition, 5-HTT (encoded by *Slc6a4*) and the vesicular monoamine transporter (encoded by *Slc18a2*) are transiently expressed in glutamatergic pyramidal neurons in early postnatal development, suggesting that 5-HT can be taken up by glutamatergic neurons and can be released upon activation[111,112]. Several genes involved in vesicular transport and downstream signaling pathways shown in this network are down-regulated in the 5-$HTT^{-/-}$ rats, suggesting reduced synaptic transmission.

RNA-seq validation by RT-qPCR

To validate the RNA-seq experiment at PND8, we selected five up- and five down-regulated genes for RT-qPCR validation in the RNA-seq samples (five per genotype) and in five independent samples per genotype. Using the RNA-seq samples, four out of the five genes downregulated in 5-HTT$^{-/-}$ rats by RNA-seq were significantly downregulated when assessed by RT-qPCR as well (*Slc6a4* ($t_{(1,4.39)}$=5.20;p<0.05); *Car4* ($t_{(1,4.07)}$=3.90;p<0.05); *Calb2* ($t_{(1,7)}$=3.93;p<0.05); *Kcnh5* ($t_{(1,7)}$=3.17;p<0.05)) and *Htr2a* showed a trend ($t_{(1,7)}$=2.18;p<0.1). Four out of the five genes upregulated in the 5-HTT$^{-/-}$ rats by RNA-seq were significantly upregulated when validated by RT-qPCR (*Htr2c* ($t_{(1,7)}$=2.60;p<0.05); *Bcl11b* ($t_{(1,3.29)}$=3.21;p<0.05); *Kctd8* ($t_{(1,5)}$=3.80;p<0.05); *Dcc* ($t_{(1,7)}$=2.83;p<0.05)) and *Scn5a* showed a trend ($t_{(1,3.07)}$=3.02; p<0.1) in the right direction (Figure 5A). We repeated the RT-qPCR in independent samples. *Slc6a4* ($t_{(1,7)}$=5.37; p<0.05), *Car4* ($t_{(1,3.22)}$=5.95;p<0.05) and *Htr2a* ($t_{(1,7)}$=2.62;p<0.05) were all significantly downregulated in the 5-HTT$^{-/-}$ rats. *Bcl11b* ($t_{(1,7)}$=5.51;p<0.05) was significantly upregulated and *Dcc* showed a trend for upregulation in the 5-HTT$^{-/-}$ rats ($t_{(1,7)}$=2.04;p<0.1). The other genes do not reach the threshold of significance, but all genes showed a change in the same direction as observed in the RNA-seq experiment (Figure 5B).

Genome-wide DNA (hydroxy)methylation

5mC and 5hmC levels were measured in mPFC DNA of five samples per genotype for each time point. No significant differences were found in 5mC levels between 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats (Figure 6A). For the 5hmC levels we found a significant increase in 5-HTT$^{-/-}$ compared to 5-HTT$^{+/+}$ rats at PND35 ($t_{(1,8)}$=6.29;p<0.05) (Figure 6B). In addition, 5hmC levels increased during development from 0.38% 5hmC/G at PND8 to 0.71% 5hmC/G at PND70 in both genotypes. Validation of the increase in 5hmC levels at PND35 was performed in an independent group of biological replicates (group 3). In this group, 5hmC levels in mPFC tissue were again increased in the 5-HTT$^{-/-}$ rats compared to the 5-HTT$^{+/+}$ rats ($t_{(1,18)}$=2.28;p<0.05) (Figure 6B). No significant expression changes were observed in genes involved in DNA (hydroxy)methylation (*Tet* and *Dnmt* genes) at PND35, nor at the other time points (Supplementary Figure 1).

Discussion

In this study, we demonstrate that a lack of functional 5-HTT affects postnatal gene expression in the mPFC particularly during the first postnatal week (PND8, earliest time point measured), with 615 differentially expressed genes between 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats for PND8 and less than 140 differentially expressed genes for the other time points (PND14, 21, 35 and 70). GO analysis showed that genes differentially expressed in the mPFC of HTT$^{-/-}$ rats at PND8 are mainly involved in neurotransmission and neurodevelopmental processes, while GO-terms enriched at other time points are less specific for neuronal-related processes. Furthermore, several genes involved in myelination show a decrease in expression in the 5-HTT$^{-/-}$ compared to the 5-HTT$^{+/+}$ mPFC at multiple ages across development.

Expression of *Slc6a4* (encoding for 5-HTT itself) is in general low in the mPFC

because the cell soma (where most of the RNA translation takes place[113]) of the serotonergic neurons is located in the raphe nuclei, while the axons (containing 5-HTT protein) reach the mPFC. We do see higher levels of *Slc6a4* mRNA at early postnatal time points (PND8 and PND14), which can be explained by the fact that at early postnatal stages 5-HTT is not only expressed by serotonergic neurons from the raphe nuclei, but also by other types of neurons

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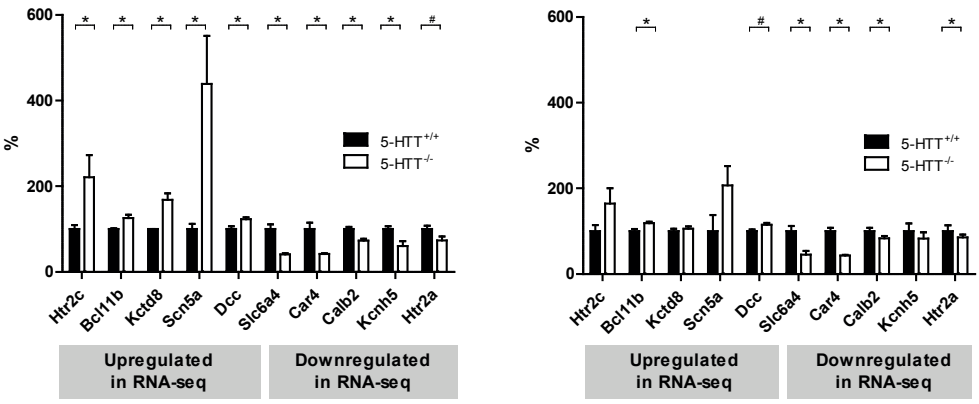

Figure 5. Medial prefrontal cortex RNA expression levels in 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats by RT-qPCR. Validation of RNA-seq results by quantitative RT-PCR (RT-qPCR) analysis in RNA-seq samples (A) and in independent biological replicates (B). RT-qPCR analysis was performed on medial prefrontal cortex RNA of 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats sacrificed at PND8. Based on RNA-seq data, five genes upregulated (left side in figure) and five genes downregulated (right side in figure) in 5-HTT$^{-/-}$ samples compared to 5-HTT$^{+/+}$ samples were selected for validation. Data are normalized for *Gabbr1* RNA levels and are presented as mean + S.E.M. of relative gene expression (% of 5-HTT$^{+/+}$ group). *p<0.05 #p<0.1.

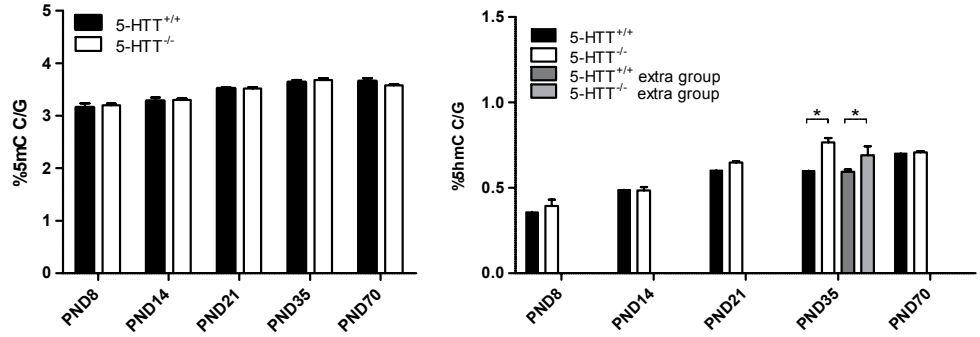

Figure 6. 5(h)mC measurements in medial prefrontal cortex DNA of 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats. (A) 5mC levels in 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats measured at five time points (n=5/genotype/time point). 5mC levels were calculated as a concentration percentage ratio of % 5-methyl-2'-deoxycytidine/2'-deoxyguanosine (%mdC/dG). (B) 5hmC levels in 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats measured at five time points (n=5/genotype/time point). 5hmC levels were calculated as a concentration percentage ratio of % 5-hydroxymethyl-2'-deoxycytidine/2'-deoxyguanosine (%hmdC/dG). 5hmC levels at PND35 were validated using an independent group of rats, depicted as 5-HTT$^{-/-}$ extra and 5-HTT$^{+/+}$ extra (n=10/genotype). *p<0.05.

in, amongst others, the mPFC (as mentioned in the introduction)[14]. We see a decrease in expression of the mutated *Slc6a4* gene in the 5-HTT$^{-/-}$ mPFC at PND8, most likely because the RNA containing the point mutation is degraded by nonsense mediated decay[114].

PCA and cluster analysis demonstrated that 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ samples cluster together per age, showing that the differences between the developmental time points were larger than the differences between genotypes. The stronger difference between time points is not surprising given the tremendous changes in the brain that occur from the early postnatal period up to adulthood compared to the relatively mild phenotype observed in the 5-HTT$^{-/-}$ rats. We also observed a clear gene expression separation between early (PND8, 14 and 21) and late (PND35 and 70) ages in the PCA, possibly indicating a genetic switch from PFC development at early postnatal life to maintenance of neural networks in adulthood. This shift from development to maintenance between PND21 and 35 was shown in our wild-type PFC samples[67] and coincides with the sharp increase of synapse formation during early brain development, which stabilizes around adolescence[32,115]. However, per time point we do see differences in gene expression between 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ mPFC samples. There are 123 genes showing differential expression at more than one time point, amongst others the myelin-related genes *Cldn11*, *Plp1*, *Mbp* and *Mag*. Overall we see a decrease in expression of myelin genes in 5-HTT$^{-/-}$ compared to 5-HTT$^{+/+}$ rats, potentially resulting in decreased or abberant myelin sheats, which fits with our previous work showing that a lack of functional 5-HTT (blocking 5-HTT by selective serotonin reuptake inhibitors) early in life results in down-regulation of genes linked to myelin[51] as well as with work of others showing a disturbed myelin sheath formation after blocking 5-HTT perinatally[82]. Why we observe an opposite effect at PND14 is unclear. In both 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats the highest expression of myelin-related genes was observed around PND21. It has been documented that myelin formation in the brain starts around PND10 in the rat and that the maximum rate of myelin accumulation occurs around PND20. Myelin accumulation does continue into adulthood, albeit at a decreasing rate[116,117]. This matches with the peak of mRNA expression we see around PND20.

Most differences in mPFC gene expression between 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats were found at PND8. At PND8 we found mainly GO-terms and pathways involved in cell migration, neurotransmission and signal transduction (See Table 1 and 2). **Cell migration**, a process important for proper formation of cell layers and cell connections in the brain, is in line with literature showing that 5-HT is involved in migration of neurons and connectivity in the brain[9,10,118]. In addition, studies have shown that cell migration and connectivity are disturbed in 5-HTT$^{-/-}$ mice and S-allele carriers, respectively[10,20]. Furthermore, cell migration is affected in 5-HT-related neuropsychiatric disorders as autism spectrum disorder and bipolar disorder[119]. In the neurotransmission-related GO-terms and signal transduction pathways enriched at PND8, several genes involved in synthesis and transmission of glutamate were found. The differentially expressed 5-HT$_{2A}$ receptor is a serotonergic receptor present on glutamatergic pyramidal cells in deep cortical layers (V and VI) in the PFC, which, upon activation, can trigger glutamate release from the pyramidal cells (Figure 4)[84]. In addition, studies have shown that

5-HTT (encoded by *Slc6a4*) and the vesicular monoamine transporter (encoded by *Slc18a2*), which are both differential expressed at PND8, are transiently expressed on glutamatergic pyramidal neurons in early postnatal development, suggesting that 5-HT can be taken up by glutamatergic neurons and can be released upon activation[111,112]. Extracellular glutamate can activate glutamate receptors on layer V pyramidal cells[84]. The down-regulation of several genes involved in vesicular transport of neurotransmitters and in downstream signaling pathways in the 5-HTT$^{-/-}$ rats suggests a reduction in synaptic transmission in these rats. During postnatal development NMDA receptors (encoded by, amongst others, *Grin2a*) and AMPA receptors (encoded by, amongst others *Gria4*) are involved in neurite outgrowth and the formation, stabilization, maturation and elimination of synapses[120-125]. Although the involvement of mGlu receptors (encoded by amongst others, *Grm2* and *Grm4*) in such processes is largely unknown, it has been recently suggested that they may also participate in synapse-stabilizing responses[126]. This suggests that altered expression of these glutamate receptors in the early postnatal PFC, as shown in our RNA-seq experiment, result in dysregulation of neurite outgrowth and synaptogenesis. Whether this dysregulation at PND8 can also result in neuropsychiatric disorders later in life is not clear. However, it has been shown that alterations in the serotonergic 5-HT$_{2A}$ and glutamatergic receptors are associated with neuropsychiatric disorders. Interactions between *Htr2a* polymorphisms and *Slc6a4* polymorphisms or SSRI treatment (blocking *Slc6a4*) were shown to predict treatment response in anxiety and depression[127-131]. Furthermore, studies showed that 5-HT$_{2A}$ receptor signaling is reduced in adult 5-HTT$^{-/-}$ mice which show depression-like behavior[95,96]. Altered *Grin2a* expression is linked to depression-like behavior in rats[132]. In addition, the mGlu2 receptor, encoded by *Grm2*, has been shown to be altered in both animal models of depression and in postmortem brain tissue of subjects with major depressive disorder[133,134]. Recent studies have shown that 5-HT$_{2A}$ and mGlu2 receptors can form a functional receptor heterocomplex, which is shown to be dysregulated in the frontal cortex of schizophrenic patients[93,97,104,135].

Another Ingenuity pathway showing a strong enrichment at PND8 is **hepatic fibrosis / hepatic stellate cell activation**, which contains, amongst others, several collagen genes. Upon activation hepatic stellate cells secrete collagens resulting in liver fibrosis[136]. There is indeed evidence that 5-HT plays a role in hepatic stellate cell function[137]. Others also showed that serotonin can enhance collagen production[138], suggesting that collagens might be a downstream target of the serotonin pathway. In the brain collagen genes play a role in e.g. axonal growth and guidance and formation of the neuromuscular junction during neurodevelopment[139]. To our knowledge we are the first to describe a link between 5-HT and collagens in the brain.

The differences found in gene expression between 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats might influence (neuro)development and behavior. We previously demonstrated that the 5-HTT$^{-/-}$ rats show a delay in development[30]. One of the main differences we observed was reduced motor coordination and muscle strength in the 5-HTT$^{-/-}$ rats in infancy. The underlying mechanism causing this aberrant development is still unknown, but it might be caused by disturbed gene

expression. In our Ingenuity Top Diseases and Bio Functions list from PND8 (Supplementary Table 5) we see the highest enrichment for genes linked to **neurological diseases** and also a high enrichment for **skeletal and muscular disorders**. Within the **neurological diseases** category we see only motor and muscle related diseases in the top three of the list, namely **seizure disorder**, **movement disorders** and **dyskinesia** (Supplementary Table 6). This suggests that several differentially expressed genes at PND8 might be associated with the defects in muscle and motor function we found in young 5-HTT$^{-/-}$ rats. From the genes we used for qPCR validation, *Htr2a, Htr2c, Scn5a, Bcl11b, Dcc, Car4* and *Slc6a4* are linked to the Ingenuity term **movement disorders**[140-146]. Studies have shown that regulation of expression of the serotonergic 5-HT$_{2A}$ receptor gene (*Htr2a*) can influence motor control[147,148]. *Htr2a* promoter methylation, associated with reduced expression, is linked to a reduced quality of movement in infants[147]. In addition, polymorphisms in the *Htr2a* gene are linked to movement related diseases like tardive dyskinesia[143] and Parkinson's disease[149]. We have to keep in mind that we focused on expression changes in the mPFC, which is not a main motor control region[35]. Nonetheless, gene expression changes observed in the mPFC do not have to be PFC-specific. More functional research (e.g. in muscles or the motor cortex) is needed to elucidate the link between the expression of these genes and the behavioral differences found in 5-HTT$^{-/-}$ rats.

We have to keep in mind that 5-HTT expression starts prenatally. Therefore, the expression differences found in the 5-HTT$^{-/-}$ rats postnatally can be a secondary effect of the changes occurring prenatally. Gene expression at postnatal stage can be different in the 5-HTT$^{-/-}$ rats compared to the 5-HTT$^{+/+}$ rats due to a different cell composition of the punch area caused by prenatal effects on developmental processes, e.g. cell migration. In addition, the expression differences in the 5-HTT$^{-/-}$ rats at postnatal time points can be a compensatory mechanism for the expression changes occurring prenatally. Genome-wide gene expression data from prenatal stages would give valuable new insights.

We did not find any differences in global 5mC in the mPFC, but we found an increase in 5hmC in the mPFC across development. This is consistent with previous findings showing that 5hmC is highly abundant in the brain[150] and levels increase during pre- and postnatal neurodevelopment[55,151]. DNA methylation plays an important role in neurogenesis, neuronal maturation and plasticity[53,54] and is linked to 5-HT-related diseases such as depression and anxiety[64,152]. So far, only one study showed that 5-HT can influence DNA methylation; 5-HT induces methylation of a conserved CpG island in the promoter region of the *CREB2* gene, serving synaptic plasticity[153]. We observed a significant increase in global 5hmC in 5-HTT$^{-/-}$ compared to 5-HTT$^{+/+}$ rats at PND35. However, the difference in 5hmC between 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats at PND35 does not coincide with differences in gene expression; we did not observe a sudden increase in the number of differentially expressed genes between the 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats at PND35. Also no differences in *Tet1, Tet2* and *Tet3* expression were observed (Supplementary Figure 1). Further research is needed to unravel the exact consequences of increased 5hmC during adolescence. In addition, it should be noted that we measured 5(h)mC at a global level, which means that methylation changes on specific genes

can still be present despite the lack of an overall effect.

In conclusion, we provide a comprehensive overview of differentially expressed genes in the mPFC of 5-HTT$^{-/-}$ rats compared to 5-HTT$^{+/+}$ rats across postnatal development. This dataset can be used to study potential new targets for treatment of 5-HT-related disorders. We showed that reduced functional 5-HTT expression affects gene expression in the mPFC mainly during early postnatal life (PND8). These changes in gene expression can affect neurodevelopmental processes resulting in aberrant brain wiring which can influence behavior during infancy, but also can result in lifelong consequences on brain functioning potentially leading to neuropsychiatric disorders in adulthood. While mainly adult rats were used to unravel the mechanisms behind 5-HT-related neuropsychiatric disorders, we propose that studying early-life stages might give more insight in the developmental dysregulation that might predispose to 5-HT-related neuropsychiatric disorders in adulthood.

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Supplementary information

Supplementary Table 1. Primers used for quantitative RT-PCR

Gene symbol	Ensembl number	Forward primer	Reverse primer
Htr2c	ENSRNOG00000030877	ACGCACGTGGTGTTATTCAC	ATGAAGATTGCCATCGTTTG
Slc32a1	ENSRNOG00000015393	CACGACAAACCCAAGATCAC	GAGGATGGCGTAGGGTAGAC
Kctd8	ENSRNOG00000026472	GACGACAAGATCTGGAGCAG	GCAGGAAGTCCCACTCTCAC
Scn5a	ENSRNOG00000015049	TTCTGGATGATGTCGGAGAG	TGCGCCACTATTACTTCACC
Dcc	ENSRNOG00000033099	GTCTGGGAGAGGAACCTCAG	CAGACACCATAAACCGGATG
Slc6a4	ENSRNOG00000003476	CTGTGGGTGTTTCAGGAGTG	GCTACTGCATAGGGATGTCG
Car4	ENSRNOG00000002916	TCGTTGGCTATGACCAAAAG	CAGGTGTAACTGTATGGCCTTG
Calb2	ENSRNOG00000016977	GCCATCTCAATTTTCCCATC	AAGGCCAGGAAAGGTTCTG
Kcnh5	ENSRNOG00000009542	CACATACCAGGAGCACAAGG	CAATGCCTTTGAAAATGTGG
Htr2a	ENSRNOG00000010063	GTTTCCTTGTCATGCCTGTG	TGATGGATGCCGTAGAAAAG
Gabbr1	ENSRNOG00000000774	TTTTCAGCCGCTTGGTTAG	ACATCACCACGGAGATTGTC

Supplementary Table 2 will be available online after publishing

Supplementary Table 3. Differentially expressed genes (PND8) linked to three GO-terms: Transmission of nerve impulse, cell- cell signaling and synaptic transmission

Gene	Log2 Ratio	Fold change	p-value	FPKM
Cartpt	0.90	1.86	5.00E-05	42.233
Cplx3	-0.73	0.60	1.00E-04	2.515
Slc6a4	-1.88	0.27	5.00E-05	1.479
Sv2b	-0.31	0.81	9.00E-04	117.867
Pnoc	0.76	1.69	8.00E-04	5.065
Vamp2	-0.27	0.83	4.65E-03	80.164
Sptbn2	-0.26	0.83	5.35E-03	195.726
Shc3	-0.42	0.75	1.00E-04	38.991
Grm2	-0.75	0.59	5.00E-05	2.862
Syn2	-0.32	0.80	1.15E-03	47.932
P2rx2	-0.48	0.72	8.80E-03	4.430
Pdyn	0.63	1.54	5.00E-05	6.293
Thbs2	0.91	1.88	5.00E-05	1.781
Hap1	0.42	1.34	5.00E-05	66.095
Gria4	0.28	1.21	4.80E-03	55.424
Gad2	1.14	2.20	5.00E-05	53.588
Dynll1	-0.28	0.82	3.20E-03	337.976
Grin2a	-0.34	0.79	3.10E-03	10.231
Gad1	0.70	1.63	5.00E-05	61.382
Gabrg1	0.27	1.21	9.60E-03	45.799
Slc17a7	-0.38	0.77	2.00E-04	165.800
Chrm1	-0.56	0.68	5.00E-05	17.832
Slc22a3	-0.76	0.59	5.00E-05	8.515
Slc5a7	0.45	1.36	1.15E-03	4.518

Supplementary Table 4. Differentially expressed genes (PND8) linked to the G protein coupled receptor signaling pathway in Ingenuity

Gene	Log2 Ratio	Fold change	p-value	FPKM	Ingeniuty pathway
Atf4	-0.31	0.80	9.50E-04	187.949	GPCR, cAMP
Chrm1	-0.56	0.68	5.00E-05	17.832	GPCR, cAMP
Dusp4	0.53	1.44	4.00E-04	13.146	GPCR, cAMP
Grm2	-0.75	0.59	5.00E-05	2.862	GPCR, cAMP, GI
Grm4	0.60	1.51	3.70E-03	3.098	GPCR, cAMP, GI
Hrh3	-0.47	0.72	4.30E-03	4.836	GPCR, cAMP, GI
Htr4	0.78	1.71	1.25E-03	3.186	GPCR, cAMP, SERT
Htr7	0.69	1.61	5.00E-05	5.535	GPCR, cAMP, SERT
Htr1f	1.03	2.04	4.00E-03	1.374	GPCR, cAMP, GI
Mc4r	0.97	1.96	6.50E-04	2.759	GPCR, cAMP
Npr3	-0.30	0.81	9.25E-03	25.496	GPCR, cAMP, GI
Npy1r	-0.38	0.77	8.00E-04	18.629	GPCR, cAMP, GI
Oprk1	1.00	1.99	5.00E-05	2.150	GPCR, cAMP, GI
Pde8b	0.56	1.48	4.50E-04	5.496	GPCR, cAMP
Rgs4	-0.52	0.70	5.00E-05	146.125	GPCR, cAMP, GI
Htr2a	-0.38	0.77	1.10E-03	11.042	GPCR, SERT
Htr2c	1.47	2.76	5.00E-05	19.846	GPCR, SERT
Sos2	-0.40	0.76	1.00E-04	16.180	GPCR, GI
Adra1d	-0.70	0.62	5.00E-05	2.751	GPCR
Prkcb	-0.28	0.82	1.80E-03	186.166	GPCR

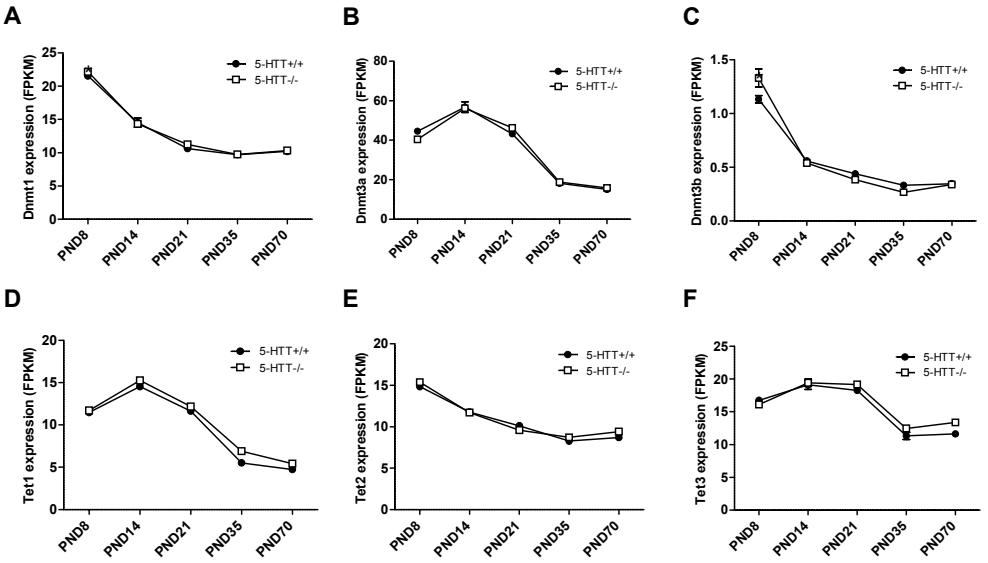
Abbreviations: GPCR = G protein coupled receptor signaling, cAMP = cAMP mediated signaling and SERT = serotonin mediated signaling

Supplementary Table 5. Top Disease and Bio Functions affected by 5-HTT$^{-/-}$ in the medial prefrontal cortex

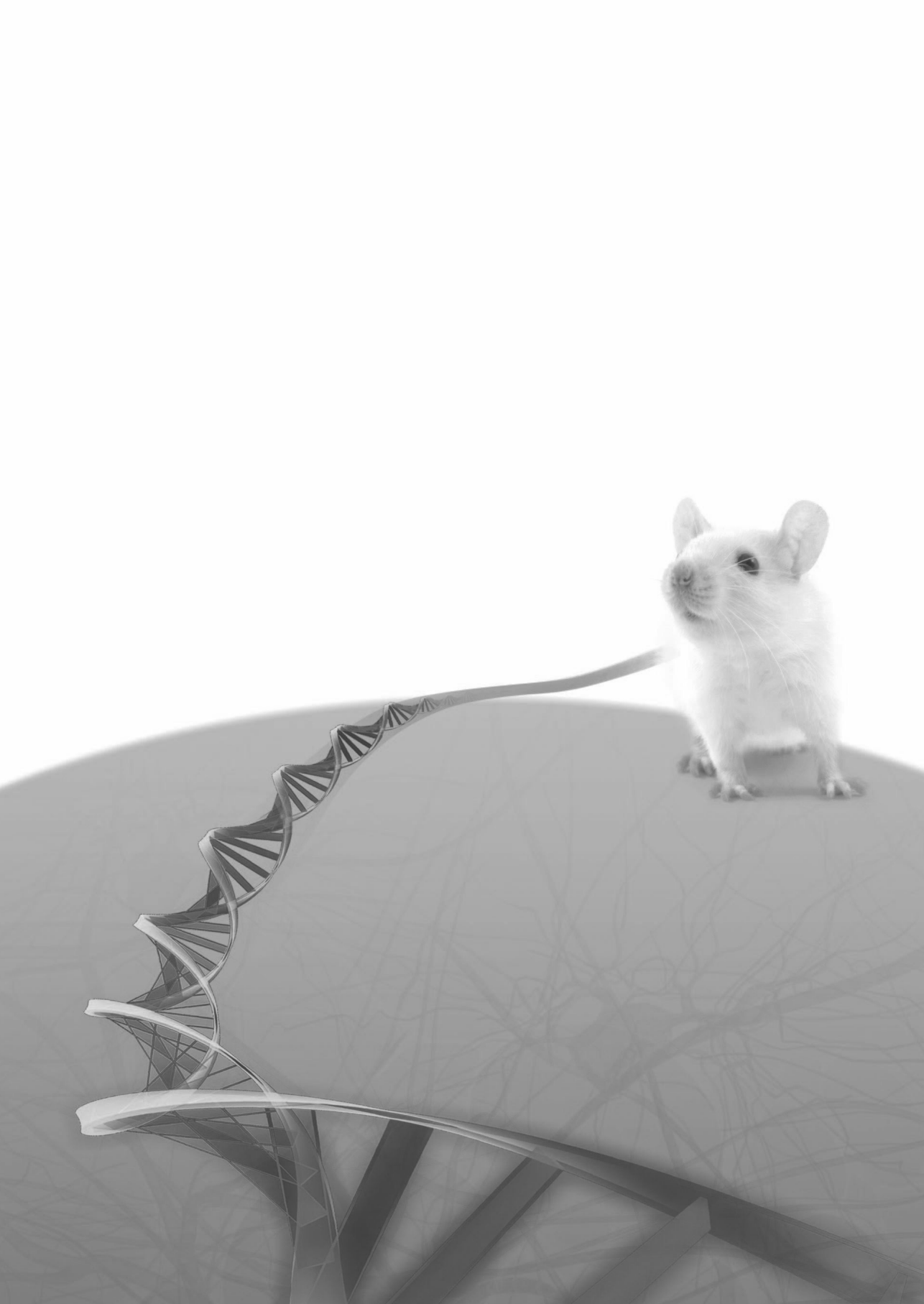
	Pathway	Top p-value	Genes
PND8	Neurological Disease	8.13E-14	142
	Psychological Disorders	2.03E-12	103
	Hereditary Disorder	2.37E-12	71
	Skeletal and Muscular Disorders	2.37E-12	106
	Cancer	1.83E-11	409

Supplementary Table 6. Top Neurological Diseases affected by 5-HTT$^{-/-}$ in the medial prefrontal cortex

	Pathway	p-value	Genes (nr and examples)
PND8	Seizure disorder	8.13E-14	*46 (e.g. Kcnh3, Kcnj9, Galr1, Gng3, Pnoc)*
	Movement Disorders	1.55E-13	*69 (e.g. Htr2a, Htr2c, Bcl11b, Dcc, Scn5a)*
	Dyskinesia	1.34E-12	*44 (e.g. Hpca, S100a10, Grin2a, Sst, Tnnt1)*
	Seizures	1.38E-12	*40 (e.g. Kcnh3, Kcnj9, Galr1, Gng3, Pnoc)*
	Disorder of basal ganglia	2.03E-12	*50 (e.g. Syn2a, Penk, Gpr88, Ptpn3, Hpca)*



Supplementary Figure 1. Expression of genes involved in DNA methylation. A-C: Expression of DNA methyltransferases (Dnmt) across mPFC development. *Dnmt2* gene is not annotated in the used reference genome (rn4). D-F: Expression of Ten-eleven translocation (*Tet*) methylcytosine dioxygenases across mPFC development. Y-axis shows expression values in fragments per kilobase per million mapped reads (FPKM). Data are presented as mean ± S.E.M. of FPKM.



7

General discussion and future perspectives

Thesis objective

Affective disorders are among the leading causes of burden worldwide. Aberrant serotonin (5-HT) signaling is involved in several affective disorders and drugs used for the treatment of affective disorders are often targeting the 5-HT system. It is known that 5-HT is involved in pathophysiology of affective disorders, but it is still unclear how (changes in) 5-HT signaling can affect behavior. Better understanding of the pathways affected by 5-HT will provide new insights in affective disorders, the working mechanism of drugs targeting 5-HT and might lead to potential new therapeutic targets. The aims of my thesis were to unravel the molecular processes occurring across postnatal medial prefrontal cortex (mPFC) development and to unravel the behavioral and molecular consequences of aberrant 5-HT signaling across development in the absence of adverse environmental conditions.

In **chapter 2** we summarized the existing literature about the effects of selective serotonin reuptake inhibitor (SSRI) treatment, resulting in increased extracellular 5-HT levels, on gene expression in the brain. The main groups of genes affected by SSRIs are genes encoding 5-HT receptors, components of non-serotonergic neurotransmitter systems, neurotrophic factors, hypothalamic hormones and inflammatory factors. To study the long-term effects of SSRIs we performed a genome-wide gene expression analysis in the hippocampus of rats 40 days after chronic fluoxetine treatment (**chapter 3**). We showed that mRNA levels of myelination-related genes were significantly upregulated in the hippocampus of SSRI-exposed rats compared to vehicle-exposed rats. In addition, the paradoxical effects of SSRI exposure during early-life and adulthood on adult anxiety and depression-like behavior would suggest that adult gene expression is affected in opposite direction after early-life SSRI exposure compared to adult SSRI exposure. In **chapter 3** we confirmed this hypothesis by showing that expression of myelination-related genes in the hippocampus of adult rats postnatally exposed to SSRIs was significantly decreased, while expression of myelination-related genes was increased after adult SSRI-exposure. In **chapter 4** we observed that both pharmacological and genetic inactivation of the 5-HT transporter (5-HTT) resulted in a developmental delay. Except for hypolocomotion, most of the observed early-life effects were normalized later in life. In **chapter 5**, we examined genome-wide expression in the developing mPFC and provided a data resource of temporal coding-gene expression, long intergenic non-coding RNA expression and alternative exon usage from infancy to adulthood. In addition, we showed that gene expression is dynamic during postnatal development and we identified a clear genetic switch from neuronal network establishment in infancy to maintenance in adulthood. This chapter provides a basis for studying mPFC-related molecular mechanisms in 5-HT related diseases. In **chapter 6**, we examined genome-wide expression in the developing mPFC of 5-HTT$^{-/-}$ rats compared to 5-HTT$^{+/+}$ rats at five developmental time points. We observed most gene expression changes in the mPFC during early postnatal life (postnatal day (PND)8) and found an enrichment of genes linked to neuronal and developmental processes like neurotransmission, neuropeptide signaling pathway and cell migration on PND8. In addition, we observed a global increase in 5-hydroxymethylcytosine (5hmC) in the

mPFC during development in both genotypes and a significant increase in global 5hmC in the 5-HTT$^{-/-}$ rats compared to 5-HTT$^{+/+}$ rats on PND35, which might influence gene expression.

Processes affected by blocking 5-HTT during adulthood

Decades ago SSRIs were used for treatment of affective disorders without knowing their exact mechanism of action. Nowadays there is data available from multiple studies showing that SSRIs increase extracellular 5-HT levels and that 5-HT can affect several pathways which might contribute to the therapeutic effect of SSRIs. There is strong evidence that besides 5-HT signaling other neurotransmitter systems, the immune system, neurotrophic factors and the HPA axis are affected by SSRIs. Effects are seen on both the gene expression[1] and the protein level[2-4]. Besides these pathways, we were the first to show that SSRIs can increase gene expression of myelin-related genes[5]. Others showed that expression of myelin-related genes was decreased in depressed patients[6-10], which nicely fits with the fact that treatment of depression with SSRIs results in upregulation of myelin genes. In addition, MRI studies showed alterations in white matter (composed of bundles of myelinated cell projections) in several brain regions in depressed patients which were (partly) normalized after SSRI treatment[11,12]. Non-serotonergic compounds acting on the immune system (e.g. anti-inflammatory drugs)[13], the HPA axis (e.g. CRH1 antagonists)[14,15], neurogenesis (e.g. BDNF infusion)[16] and myelination (e.g. antimuscarinic compound that enhances myelination)[17], also reduce affective behavior (mainly tested in animals), confirming that these pathways actually contribute to the treatment of affective disorders and are not just side effects of SSRIs.

Most studies focused on the short-term effect of chronic SSRI treatment (24 hours after the last SSRI treatment), but we studied the long-term effects of chronic SSRI treatment and found myelination to be affected at gene expression level 40 days after the last treatment. Effects on neurotransmitter systems, the immune system, neurotrophic factors and the HPA axis were not visible in our study and might represent acute/short term effects. However, these pathways can still be affected at other levels. For instance, protein levels could be altered, or further downstream structural changes could have taken place, which can contribute to the therapeutic effects of SSRIs. Information about the long-term effects on protein and structural changes after cessation of SSRI treatment is very limited. One study showed that chronic SSRI treatment caused a persistent decrease in brain dopamine levels for 7-14 days after cessation of treatment, depending on the dose used[18]. Others showed that chronic SSRI treatment in olfactory bulbectomized rats (model of depression) results in changes in open field activity, one and two weeks after treatment cessation[19]. Since a relatively high number of affective disorder patients experiences a relapse[20,21], it would be interesting to investigate whether long-term SSRI-induced changes, e.g. myelination changes, are normalized at the time of a relapse and whether this can be used as a potential (predictive) marker for development of a relapse. In addition, we have to be aware that the effects of SSRI treatment are influenced by several factors such as type of SSRI, brain region, treatment length, animal/strain, sex and environmental factors which might account for the

differences seen between studies.

Processes affected by blocking 5-HTT during the perinatal period

Pharmacological (SSRI exposure) and genetic (5-HTTLPR S-allele carriers, 5-HTT$^{-/-}$) reduction of functional 5-HTT during development have been associated with negative effects on development and behavior. The pathways affected by developmental 5-HTT reduction depend on the time point of SSRI exposure, the length of exposure, the type of SSRI, the brain region of interest and the time point of measuring the effect. We here discuss the effects of perinatal (pre and/or postnatal) reduction of functional 5-HTT on brain development.

The most reported early postnatal effects in children perinatally exposed to SSRIs are neonatal withdrawal symptoms, which usually disappear within 2 weeks after birth[22]. In addition, perinatal exposure is linked to teratogenic effects (e.g. congenital heart defects) and developmental delay, which in most studies reflects a delay in gross motor function milestones. Furthermore, autism spectrum disorder (ASD) is a developmental disorder that is associated with SSRI exposure during pregnancy in humans[23-25]. Also in rodents ASD-related behavior has been found after perinatal SSRI exposure[26]. The delay in social-sensory and motor development observed in perinatally SSRI-exposed rats in this thesis, and the decreased sociability found in these rats[27] are comparable to ASD-related phenotypes[28]. SSRI exposure leads to 5-HTT inhibition and thereby a rise in 5-HT levels. This condition is exacerbated in 5-HTT$^{-/-}$ rats and, accordingly, we observed that social-sensory and motor development were delayed with greater intensity in 5-HTT$^{-/-}$ rats compared to perinatally SSRI-exposed rats. Additionally, we showed that 5-HTT$^{-/-}$ rats, compared to 5-HTT$^{+/+}$ controls, displayed increased repetitive (object directed) behavior and an increase in brain/body mass index, both of which have been linked to ASD[29]. In contrast to the clinical symptoms, the early postnatal effects on gene expression or structural changes in the brain after perinatal reduction of functional 5-HTT are hardly investigated. No gene expression studies investigating early postnatal changes in S-allele carriers or after perinatal SSRI exposure are available. We investigated early postnatal gene expression in the mPFC of 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats and showed that expression of genes involved in cell migration, neurotransmission and signal transduction was affected on PND8 in the 5-HTT$^{-/-}$ rats. Whether these pathways contribute to the observed clinical effects is not clear. However, there are several links between differentially expressed genes and behavior phenotypes. Differentially expressed genes in 5-HTT$^{-/-}$ rats on PND8 were strongly enriched for genes linked to diseases with muscle and motor dysfunction (e.g. movement disorders), showing a link with the delay in development of motor functions as observed in SSRI exposure children[30-32] and in our 5-HTT$^{-/-}$ and perinatal SSRI-exposed rats. Furthermore, dysregulation of cell migration might result in aberrant brain wiring, which can influence behavior during infancy, but can also result in lifelong consequences on brain functioning. Defects in cell migration are often found in ASD[33]. In addition, within the neurotransmission gene category we found a number of genes related to glutamate signaling, which is a process

that is shown to be dysregulated in a group of ASD patients[34].

Frequently reported effects of perinatal reduction of 5-HTT observed at adulthood are anxiety and depression-like symptoms as well as substance abuse. These effects are observed in models of lifelong 5-HTT reduction such as S-allele carriers[35-37] and 5-HTT$^{-/-}$ rodents[38,39], but also in rodents exposed to SSRIs during the perinatal[27,40,41] and, to a lesser extent, adolescent[42] period, suggesting that the effects at adulthood are a consequence of developmental 5-HTT reduction. Studies investigating adult effects of early-life reduction of functional 5-HTT on gene expression and structural changes in the brain are limited. In postnatally SSRI-exposed rats we observed a decrease in the expression of myelin-related genes at adulthood. Also in the 5-HTT$^{-/-}$ rats we observed a decrease in expression of some myelin genes (*Cldn11, Plp1*) at adulthood. Based on the similarity of these results with the results in postnatal SSRI-exposed rats, we assume that in the 5-HTT$^{-/-}$ rats this effect on myelin gene expression is also caused by early-life 5-HTT inactivation. Others observed structural changes of myelin, such as a disturbed myelin sheath formation, at adulthood after SSRI exposure during brain development[43]. Furthermore, postnatal SSRI exposure was found to cause downregulation of brain derived neurotrophic factor IV (*Bdnf* IV) gene expression in the hippocampus[44] and long-term sex-dependent effects on hippocampal plasticity at adulthood[45]. Moreover *Npas4*, one of the transcription factors regulating *Bdnf* gene expression, was significantly decreased in the hippocampus of adult rats prenatally exposed SSRIs[46]. In summary, only a few studies focused on the long-term molecular and structural effects of early-life reduction in functional 5-HTT and more research is needed to understand the underlying mechanisms involved in the anxiety and depression-like behavior.

mPFC development and changes in 5-HT signaling

We investigated gene expression across postnatal development in the mPFC, which is one of the latest maturing brain regions. The mPFC has an extensive serotonergic innervation[47,48] and has been implicated in neuropsychiatric disorders like anxiety, depression and drug addiction[49-51]. In our developmental expression timeline in the mPFC of wildtype rats (chapter 5) we showed a clear switch from genes involved in neuronal network establishment in infancy to those involved in neuronal homeostasis and maintenance in adulthood. Moreover, we showed that in these wildtype rats most expression changes occur in infancy and the number of differentially expressed genes reduces toward adulthood. In addition, we found the highest number of differentially expressed genes between 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats on PND8 (chapter 6), showing that manipulating 5-HT levels causes the strongest effects in the period with the highest gene expression dynamics. As shown in chapter 5, this early postnatal period is important for synaptogenesis and network establishment and the high number of differentially expressed genes in the 5-HTT$^{-/-}$ rats in this period fits with the important role of 5-HT in developmental processes such as neuronal migration and network formation in the mPFC[52-55]. Several genes proven to be involved in prefrontal developmental events are associated with neurodevelopmental diseases and show differential expression

between consecutive time points in wildtype rat mPFC tissue (chapter 5, table 1). We were able to show that some of these genes, e.g. *Gad1* (Glutamate decarboxylase 1) and *Nrp2* (Neuropilin 2, involved in axon guidance), are differentially regulated in PND8 mPFC tissue of 5-HTT$^{-/-}$ rats compared to 5-HTT$^{+/+}$ rats as well. These findings support the existing literature about involvement of 5-HT dysregulation in neurodevelopmental diseases, such as autism spectrum disorder[26]. Furthermore, we found multiple differentially expressed lincRNAs and genes showing alternative exon usage during postnatal mPFC development in wildtype rats. Similar analysis can be done using our 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ RNA-seq data and might provide additional information about the role of 5-HT signaling on transcriptional changes across postnatal mPFC development.

Paradoxical effects of SSRIs

There is increasing evidence that early-life SSRI exposure results in paradoxical behavioral and molecular effects compared to adult SSRI exposure. While adult SSRI treatment induces antidepressive and anxiolytic effects, perinatal SSRI exposure results in increased anxiety and increased depression-like symptoms. Furthermore, teenagers and adults with ASD may benefit from SSRIs[56], while SSRI exposure during the perinatal period increased the risk for ASD[24-26]. It is likely that similar molecular pathways are affected in both cases, since adult SSRI exposure can alleviate the behavioral effects induced by adolescent[42] and neonatal[57] SSRI exposure. Indeed, studies, so far, revealed two pathways showing opposite effects, namely neurogenesis and myelination. We showed that adult SSRI exposure can increase gene expression of myelin-related genes, which might restore the reduced expression of myelin genes found in depressed patients[6]. Opposite effects were observed in adult rodents after developmental SSRI exposure, namely decreased expression of myelin-related genes (this thesis), disturbed myelin sheath formation and reduced callosal connectivity[43]. Neurogenesis is also showing paradoxical effects. Chronic administration of SSRIs in adult rodents leads to increased hippocampal neurogenesis[58] and increased expression of the neurotrophic factor BDNF[59]. However, there are also studies showing opposite or no effects[60-62]. Developmental SSRI exposure caused a decrease in hippocampal plasticity[45] and reductions in mRNA of *Bdnf IV*[44], *Npas4*, (transcription factor regulating *Bdnf)*[46] and *Erk2* and *Creb* (downstream targets of *Bdnf*)[63] at adulthood. The finding that neurogenesis and myelination are showing opposite effects suggest that these pathways are involved in the ”SSRI paradox”. By finding opposite effects on gene expression we are now one step further in understanding the opposite behavior effects, but it still remains to be elucidated why the direction of an effect is dependent on the time point of SSRI exposure. Two factors that contribute to different effects during development are the transient wide-spread expression of 5-HTT during development and the effects of 5-HT on neurodevelopmental processes such as cell proliferation, cell differentiation, neuronal migration and network formation[52-54]. The downstream pathways of 5-HT during developmental time points may differ from the pathways at adulthood in order to be able to help building the brain. These different downstream pathways potentially

account for 5-HTs neurotrophic functions, but might also be involved in the opposite effects on myelination, neurogenesis and behavior. Given the transient wide-spread expression of 5-HTT and the neurotrophic function of 5-HT, different effects during development are expected, however it is interesting, but still unclear, why several pathways show opposite effects compared to adulthood.

Depression versus SSRIs during pregnancy

The best way to investigate the risks of SSRI use during pregnancy in comparison to women who have the same severity of depressive symptoms but are not receiving SSRI treatment during pregnancy, would be conducting randomized, double-blind, placebo-controlled studies in pregnant mothers. However, this is seen as unethical due to the potentially negative impact on the fetus. As a consequence the available data about the effects of SSRI treatment and untreated depression (and also anxiety and stress[64]) on the (un)born child and the available treatment guidelines[65-67] are based on retrospective case-control studies, smaller prospective control studies and animal studies (like this thesis). This thesis increases the knowledge about the effects of SSRIs, by showing a delay in development and altered myelin gene expression at adulthood, but based on the data in this thesis alone we cannot give a good advice to pregnant mothers about SSRI use during pregnancy. Together with the available literature we can speculate about the benefits and disadvantages of SSRI exposure and maternal depression during gestation. SSRI use during pregnancy has several negative effects on the developing child, like neonatal withdrawal symptoms, developmental delay, teratogenic effects and an increased risk of developing neuropsychiatric disorders such as ASD, depression and anxiety. In contrast, depression itself can also have a major impact on the development of the (unborn) child, like preterm birth, lower birth weight, delayed cognitive and motor development, higher impulsivity, maladaptive social interactions, and behavioral and emotional difficulties[64,68,69]. Both, depression and its treatment, cause very similar negative effects on the offspring, making the decision to discontinue treatment or to wait with initiating treatment rather difficult. For both conditions most effects on the offspring are transient and only a few effects might last into childhood, adolescence and adulthood (as we also show in chapter 4). Studying long-term effects in humans is hampered by variability in e.g. depression severity, treatment period, treatment length and environmental factors. Therefore, evidence for long-term effects, e.g. anxiety and depression, is mainly based on animal studies (under well-controlled environmental conditions). The most recent meta-analyses and reviews suggest that the risks of SSRI intake during pregnancy for poor fetal outcome overall are small to moderate[70]. However, untreated depression has similar risks for fetal outcome and, in addition, untreated depression during pregnancy also increases maternal risks like hypertension, suicide and unhealthy behaviors as smoking and the use of drugs and alcohol[70,71]. Current best practice is to make a decision based on a personalized risk-benefit analysis. If mood has been stable for a long period of time discontinuation of SSRIs during pregnancy might be considered. Psychotherapy should also be considered

as treatment and might especially be beneficial for mild to moderate depression[72]. For severe (recurrent) depression psychotherapy alone is not recommended but can be used in combination with antidepressants. When using SSRIs during pregnancy it is recommended to use one drug (monotherapy), to use an SSRI with a relatively low placental transfer (e.g. fluvoxamine)[73], to not change medication during pregnancy and to use the lowest effective dose. The extent of fetal exposure during pregnancy is also relevant to those women who choose to breast feed. If a woman takes an SSRI in pregnancy, concerns about breast feeding exposure are not warranted, since pregnancy exposure is magnitudes of order greater than breast feeding exposure[67].

Potential new therapeutic options

SSRIs are currently the first line treatment for affective disorders. Adult SSRI exposure reveals favorable effects on affective disorders, but also has adverse effects, like sexual dysfunction[74], suppression of rapid eye movement sleep[75], nausea[76,77], decreased appetite[78] and aggression/violence[79,80]. In addition, it takes weeks of treatment before clinical effects are noticed. Furthermore, as mentioned above, SSRI exposure during development has many (negative) consequences on brain development and behavior. These adverse effects emphasize that further optimization of chronic treatment of affective disorders is needed.

Pathways affected by SSRIs potentially contribute to their efficacy and targeting these pathways might augment the therapeutic response or reduce adverse effects. Based on our review about the effects of SSRIs on gene expression and our RNA-seq experiment in SSRI-exposed rats, we can propose several directions for the treatment of effective disorders in adulthood, namely; 5-HT receptors, non-serotonergic neurotransmitter systems, neurotrophic factors, hypothalamic hormones, inflammatory factors and components involved in myelination. Several of these directions are already under investigation. In addition, the genes and pathways mentioned in chapter 5 (neurodevelopmental genes) and chapter 6 (genes affected by genetic inactivation of 5-HTT), can be used for development of therapeutic targets for treatment of 5-HT related diseases. One strategy to improve therapeutic response is to refine and expand on the monoamine mechanisms. Augmenting strategies are investigated for several years already and include adjuvant therapy with atypical antipsychotics, which act by their antagonistic effect on $5\text{-HT}_{2A/2C}$, and treatment with multimodal drugs targeting 5-HT receptors in combination with 5-HTT inhibition in one molecule[81]. There are a few multimodal and atypical antipsychotics drugs approved for treatment of depression and several new drugs are under investigation[81]. In addition, adjunct treatment with the slow release 5-hydroxytryptophan (5-HT precursor) in mice can safely and effectively elevate 5-HT beyond the SSRI effect and might represent a novel treatment for treatment-resistant depression[82]. Other research is focusing on mechanisms that go beyond the monoamine system. There is an increasing interest in targeting glutamate neurotransmission. The N-methyl-D-aspartate (NMDA) receptor antagonist ketamine is able to produce an immediate and long-lasting antidepressant effect in patients with treatment-resistant depression[83] and is now used as a

treatment option for this group of patients[84]. Blockade of NMDA receptors by ketamine will lead to an increase in extracellular glutamate, which is thought to mediate antidepressant effects by activating pathways involved in neuronal plasticity and synaptogenesis[85-87]. However, ketamine also has adverse effects, like psychotomimesis and abuse liability[88], which prevent its use as a first-line treatment for affective disorders. In addition, compounds targeting other glutamate receptors, e.g. metabotropic glutamate receptors[89,90], are currently investigated in clinical trials. Furthermore, compounds acting on neuroinflammation have been tested. Cyclooxygenase-2 inhibitors showed antidepressant efficacy as an add-on therapy to SSRIs[91,92] and a TNF alpha antibody showed a trend towards antidepressant activity in a group of depressed patients with elevated levels of pro-inflammatory cytokines[93]. A few compounds involved in the HPA-axis have been tested. CRF1 receptor antagonists have shown anxiolytic-like effects in animals, but showed mixed effects on antidepressant behavior and no CRF1 antagonist has successfully completed a Phase III clinical trial in humans yet[94]. Also research around neurotrophic factors is ongoing. Especially BDNF and its TrkB receptor are the focus of several studies. In rats infusion of BDNF into the dentate gyrus produced antidepressant effects[16]. No CNS drug-like molecule is available yet, but attempts to transfer BDNF or TrkB ligands across the blood-brain barrier using liposomes or by conjugation with a protein transduction domain are being made[95-98], also in our lab. Lastly, we found an increase in the expression of myelin-related genes, which fits with the findings of Aston and colleagues who reported a decrease in the expression of myelin-related genes in depressed patients[6]. Myelination would be an interesting new direction for drug development. We showed long-term effects of SSRI exposure on myelination, which suggests that changes in myelination might be more stable changes and targeting myelination might potentially reduce the chances of developing a relapse after discontinuation of SSRI treatment. So far, there is one animal study that supports the finding that increasing myelination can decrease depression-like symptoms. Oral clemastine treatment (associated with enhanced oligodendrocyte progenitor differentiation) rescued the impaired myelination in mice undergoing prolonged social isolation and reversed the depression-like social avoidance behavior in these mice[17]. More research is needed to explore the opportunities of myelinating agents as a treatment for affective disorders.

While a lot of research is focusing on new compounds for the treatment of affective disorders, none of these compounds have been tested in the developmental period yet. Testing safety and efficacy during development is important, because drugs can have a completely different effect during development as a consequence of differences in e.g. metabolism and hormone levels during development and potential detrimental effects of the drug on neurodevelopmental processes. Since developmental SSRI exposure can have negative outcomes, development of new drugs for the pediatric population and for use during pregnancy is highly needed. Since blocking 5-HTT during development can result in negative effects later in life, it may be worthwhile to explore non-serotonergic compounds, e.g. related to non-serotonergic neurotransmitter systems, neurotrophic factors, hypothalamic hormones,

inflammatory factors and myelination. Another approach would be the development of SSRIs with a lower placental transfer.

Future perspectives for studying the effects of reduced functional 5-HTT in rodents

In the paragraphs above we already proposed several interesting directions for future research. In addition, we would like to discuss a few more directions based on this thesis specifically. In our gene expression studies we chose to look at the overall effect of reduced 5-HTT in a brain region. We have investigated gene expression in the hippocampus and mPFC in a mixture of cell types. Using this approach, we did observe differences in gene expression which contribute to mPFC development and 5-HT related neurodevelopmental and behavioral phenotypes, but we do not know whether these differences are a consequence of altered gene expression within a cell type or a consequence of a different composition of cells in the investigated area. Furthermore, 5-HTT expression starts prenatally, therefore in the 5-HTT$^{-/-}$ rats the mRNA expression differences we found postnatally can be a secondary effect of the changes occurring prenatally. For example, the gene expression observed at postnatal time points can be a compensatory mechanism for the expression changes occurring prenatally and gene expression at postnatal stage can be different due to a different cell composition of the punch area caused by prenatal effects on e.g. cell migration. In addition to our gene expression data, information from prenatal stages and gene expression per cell type would give valuable new insights in the effects of a reduction in functional 5-HTT, which might help in defining potential drug targets. In order to obtain expression data per cell type, fluorescence-activated cell sorting (FACS)[99] followed by RNA-seq would be a good approach. Potential cell types to focus on would be oligodendrocytes (based on the myelin-related effects shown in chapter 3 and 6) and glutamatergic pyramidal cells (based on gene expression changes observed in the 5-HTT$^{-/-}$ rats on PND8 in chapter 6, Figure 4).

We have shown several processes that are dysregulated on gene expression level by a reduction in functional 5-HTT. It would be relevant to investigate whether these processes are dysregulated on protein or structural level as well. For instance, investigating myelin protein expression or myelin structure by performing immunohistochemistry and electron microscopy, respectively. In addition, it would be interesting to investigate whether part of the gene expression observed in 5-HTT$^{-/-}$ rats can be normalized by injecting 5-HTT constructs into 5-HTT$^{-/-}$ rat embryos by *in utero* electroporation. With this technique various constructs, either up- or downregulating the gene of interest, can be microinjected into the lateral ventricles of the developing embryonic brain. The construct is then incorporated into the ventricular surface by electroporation using a series of unipolar square wave pulses, driving expression into specific brain areas defined by the position of the electrodes[100]. Besides normalizing 5-HTT itself, this technique can also be used to investigate whether normalizing one or combinations of the differentially expressed genes can rescue a particular behavior defect.

Another direction for follow-up research is looking into epigenetic changes caused by altered 5-HTT expression. Up to now we only investigated global DNA (hydroxy)methylation (5(h)mC) changes, but it would be interesting to also look at genome-wide, gene-specific, 5(h)mC in 5-HTT$^{-/-}$ rats or in SSRI-exposed rats since aberrant DNA methylation patterns in the brain, in saliva and in blood are linked to affective disorders[101-105]. Investigating genome-wide 5(h)mC differences in the 5-HTT$^{-/-}$ rats and analyzing whether these changes fit with the observed gene expression changes will result in valuable new information about the downstream effects of 5-HT signaling and the working mechanisms of SSRIs. In addition, histone modifications have been linked to affective disorders, especially acetylation[106-110]. Studying histone modifications using chromatin immuno-precipitation followed by sequencing (ChIP-seq) in our 5-HTT$^{-/-}$ rats will complement the gene expression (and DNA methylation) data.

Performing the same gene expression and behavior experiments in 5-HTT$^{+/-}$ rats, which more resemble the S-allele carriers in terms of 5-HTT protein expression, would be of great interest as well. So far results in the 5-HTT$^{+/-}$ rats are mixed, which might depend on the environmental conditions (see below). They sometimes behave like the 5-HTT$^{-/-}$ rats[111] and sometimes their behavior is similar as observed in the 5-HTT$^{+/+}$ rats[112].

Furthermore, models of reduced 5-HTT expression (5-HTT$^{-/-}$, 5-HTT$^{+/-}$, SSRI exposure) have been associated with increased susceptibility to environmental conditions, which can result in favorable effects when exposed to positive environmental conditions and may result in negative effects when exposed to negative environmental conditions (e.g. stress)[113-115]. Positive effects observed in adult S-allele carriers and 5-HTT$^{-/-}$ rodents are enhancement of cognitive functions, including better decision-making skills[116-118], inhibitory control[112], and reversal learning[119,120]. Prenatal SSRI exposure is associated with improved spatial learning[121]. So far these studies focused on adult rats. Are there favorable effects in models of reduced 5-HTT expression during development as well? Investigating the influence of stress is beyond the scope of this thesis (investigating the effects of dysregulated 5-HT signaling in the absence of adverse environmental conditions), but it has been shown by others that stress, as a negative environmental condition, can differentially effect adult behavior and gene expression in models of reduced 5-HTT expression compared to non-stressed groups[122-124]. Investigating whether exposure of 5-HTT$^{-/-}$ and SSRI-exposed rats to negative environmental conditions, such as stress, results in differential effects on behavior and gene expression during development, compared to non-stressed conditions, might be a possible future direction.

Conclusion

Overall with this thesis we have come to a further understanding of the effects of 5-HT on behavior and gene expression across development. In **chapter 4** we extended the studies from Olivier and colleagues[27,38], which showed effects of perinatal SSRI exposed rats and 5-HTT$^{-/-}$ rats on adult behavior, by showing a delay in development in perinatal SSRI exposed

rats and 5-HTT$^{-/-}$ rats, with the strongest effects on motor behavior. On the transcriptional level, we explored genome-wide transcriptional changes in the wildtype medial PFC during development (**chapter 5**) and we were the first to investigate changes in gene expression in this brain region in the 5-HTT$^{-/-}$ rats across development (**chapter 6**), resulting in datasets which can be used as a resource for investigators interested in the basic developmental processes of the mPFC, the mechanisms of neurodevelopmental and neuropsychiatric disorders and for selecting potential new targets for treatment of 5-HT-related disorders. We found several pathways to be affected in the 5-HTT$^{-/-}$ rats on PND8, including genes involved in motor diseases, which complement the observed delay in motor behavior in these rats. In **chapter 2** we summarize the existing literature about the effect of SSRIs on gene expression and concluded that information about the long-term effects of SSRIs (>24 hours after last exposure) and about gene expression changes by perinatal SSRI exposure was lacking. In **chapter 3** we focused on these gaps and showed that myelin genes are upregulated 40 days after the last SSRI exposure. In addition, we showed that SSRI exposure results in opposite effects on expression of myelination-related genes after perinatal SSRI exposure, which correlated with (opposite) anxiety and depression-like behavior and brings us a step further in unraveling the mechanisms behind the frequently reported paradoxical effects of perinatal versus adult SSRI exposure on behavior[125] .

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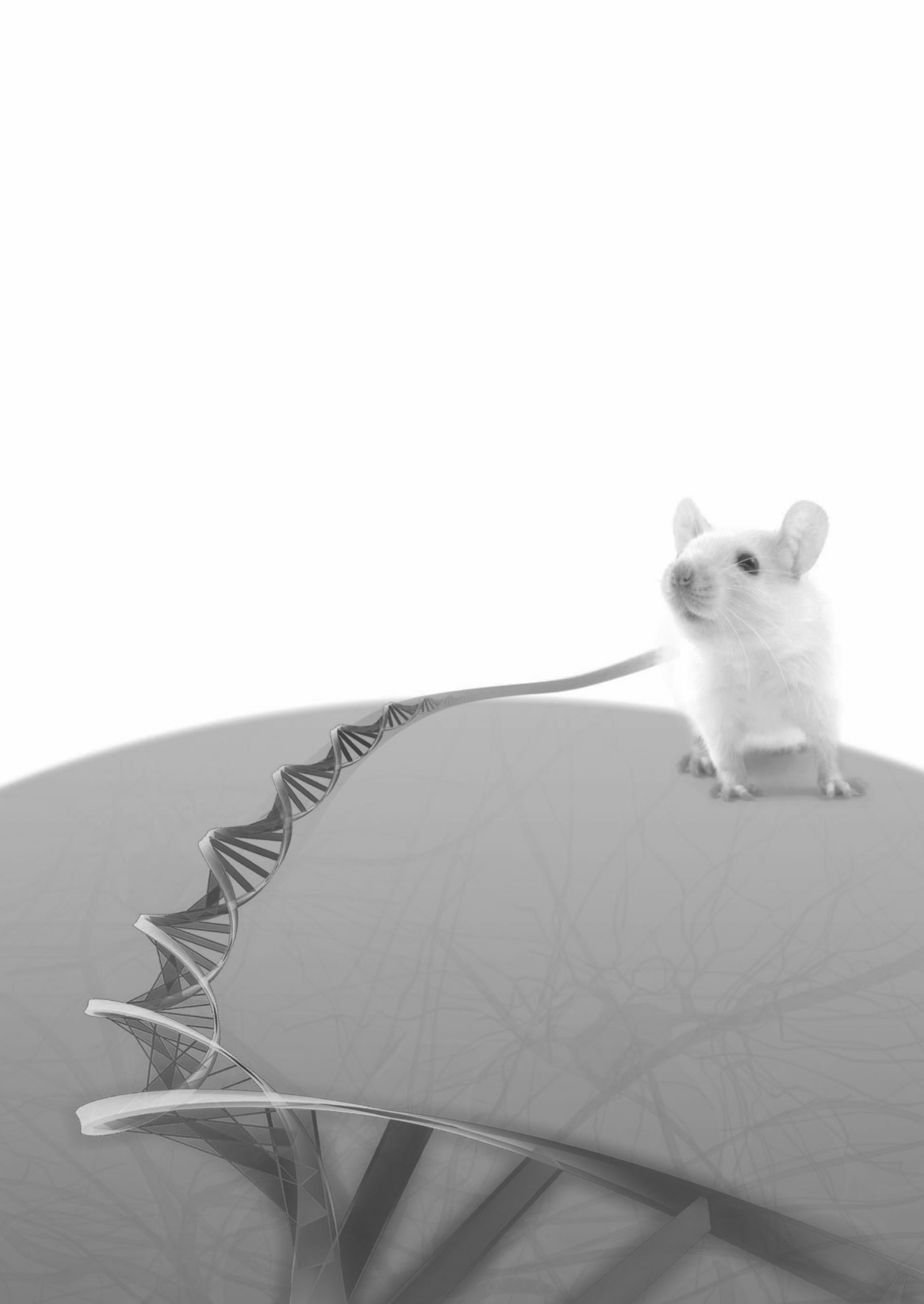


8

English summary
Nederlandse samenvatting

English Summary

Affective disorders are psychiatric disorders characterized by disturbances in mood and emotions in such a way that normal functioning is hampered. Examples of such disorders are depression, anxiety-related disorders and bipolar disorders. Affective disorders are frequently occurring diseases caused by a combination of genetic and environmental factors. An important molecule involved in affective disorders is serotonin (5-hydroxytryptamine, 5-HT). Serotonin is a neurotransmitter (a messenger which transmits information between neurons) produced in serotonergic neurons which plays an important role in processes such as mood, sleep, sexual activity, emotion and appetite. Serotonin is released in the extracellular space via vesicles and can bind to serotonin receptors present on other neurons, and activate a variety of downstream processes. Serotonin transporters (5-hydroxytryptamine transporter, 5-HTT) present on the serotonergic neurons regulate the amount of available extracellular serotonin by facilitating the reuptake of serotonin in the neuron. Decades ago it was shown that low extracellular serotonin levels in the brain are associated with affective disorders, such as depression. Furthermore, studies showed that genetic variations (such as polymorphisms; natural variations in the DNA sequence which occur with relative high frequency in the population) in serotonin genes are associated with affective disorders. A well-studied polymorphism is the 5-HTT-linked polymorphic region (5-HTTLPR) polymorphism located in the part of the DNA that regulates the expression of the serotonin transporter. The short variant (S-allele; contains less repeats of a repetitive sequence) is associated with lower levels of the serotonin transporter and people carrying this variant have an increased risk of developing depression and anxiety-related disorders.

The serotonin system is an important target for a variety of drugs prescribed for treatment of affective disorders. The most frequently prescribed drugs for the treatment of affective disorders are selective serotonin reuptake inhibitors (SSRIs). SSRIs block the serotonin transporter and thereby the reuptake of serotonin in the neuron, resulting in increased extracellular serotonin levels. SSRIs are mostly known as antidepressants, but are also prescribed for, amongst others, anxiety-related disorders. Despite many positive effects, clinical studies also showed several adverse effects of SSRIs, namely nausea, reduced appetite, aggression/violence, suppression of rapid eye movement (REM) sleep and sexual dysfunction. Another disadvantage is that it often takes weeks of treatment with SSRIs before clinical effects are noticed.

The highest risk for women to suffer from depression is during their childbearing years. To treat depressed pregnant women, SSRIs are frequently prescribed. Studies have shown that SSRIs can reach the unborn child by crossing the placenta and that SSRIs can be transferred by breastfeeding. It has been shown that this early-life SSRI exposure can have negative effects on the development of the child, such as preterm birth, low birth weight, neonatal withdrawal symptoms, defects in motor behavior, autistic-like behavior, increased blood pressure in the lungs (pulmonary hypertension) and congenital heart defects. In addition, mainly in animal studies it is shown that perinatal SSRI exposure increases the risk

of developing anxiety and depression-like symptoms in adulthood. It is striking that SSRI exposure during development is associated with an increased risk of developing anxiety and depression-like symptoms, while exposure in adulthood suppresses these symptoms (age-dependent effect). It is important to mention here that untreated affective disorders of the mother can also have negative consequences on the (unborn) child. In summary, SSRIs show age-dependent effects, with negative outcomes after early-life exposure. Of special interest is the opposite age-dependent effect on anxiety and depression-like behavior. In addition, SSRI use in adulthood can result in several adverse effects, despite their positive effect on anxiety and depression-like behavior. Taken together, this shows that increasing the knowledge about the cause of affective disorders and the effects of serotonin and SSRI exposure in adulthood as well as during development is necessary to improve treatment of affective disorders.

In this thesis we focused on the molecular changes occurring in the brain as a consequence of increased extracellular serotonin levels during postnatal development as well as in adulthood. In addition, we looked into the behavioral effects of increased extracellular serotonin levels from early postnatal age till adulthood.

In chapter 2, we summarized the literature available at the beginning of my PhD about the effects of SSRI treatment, resulting in increased extracellular 5-HT levels, on gene expression in the brain. The majority of the genes affected by SSRIs are genes encoding 5-HT receptors, components of non-serotonergic neurotransmitter systems, neurotrophic factors (factors which stimulate cell division, development and survival), hypothalamic hormones and inflammatory factors. We noticed that most studies focused on gene expression 24 hours after the last SSRI exposure, while research about the long-term effects of SSRIs can bring valuable additional information about the working mechanisms of SSRIs. In addition, there is hardly any information about the effects of perinatal SSRI exposure on gene expression. Therefore, in chapter 3 we focused on the long-term effects of SSRI exposure in adulthood and around birth on gene expression in the hippocampus. The hippocampus is a brain region implicated in psychiatric disorders and mainly involved in memory formation. We demonstrated in adult rats that the expression of several genes involved in myelination is increased in the hippocampus 40 days after the last SSRI administration in comparison to a control group that was not treated with SSRIs. Myelination is the formation of myelin around axons, which enhances the speed of the transmission of information encoded in action potentials. Furthermore, we showed a negative correlation in these rats between the expression levels of myelin genes and anxiety levels investigated with a behavior test; the increased expression of myelin genes correlated with lower anxiety levels. It would be interesting to investigate whether the increased gene expression will decline at the time of a relapse and whether myelination can be used as a potential (predictive) marker for development of a relapse. In addition, we showed in this chapter that postnatal SSRI exposure results in a decrease in expression of myelin genes in the hippocampus when the rats are grown-up. This gene expression also shows a negative correlation with the level of anxiety; a lower expression of myelin genes correlates with increased anxiety levels. In summary,

expression of myelin genes is increased after adult exposure and decreased after perinatal exposure. This opposite age-dependent effect of SSRI exposure on expression levels of myelin genes fits very well with the opposite effects of perinatal versus adult SSRI exposure on anxiety and depression-like behavior which have been frequently described in the literature. In chapter 4, we investigated the effects of changes in the serotonin system, namely increased extracellular serotonin levels, on development of behavior. There are already several early behavioral effects of SSRI exposure on young age described in the literature, but the effects during later development of the animals were still unknown. Furthermore, there were no studies that have investigated behavior across developmental stages in 5-HTTLPR S-allele carriers. To elucidate this, we used rat models and we observed that rats perinatally exposed to SSRIs as well as rats lacking the serotonin transporter their whole life (so-called serotonin transporter knockout rats, a model for 5-HTTLPR S-allele carriers) show a delay in development, especially on motor coordination and reflex development. With the exception of hypolocomotion, most behavior differences where normalized around adolescence. Around adolescence and adulthood, we observed reduced interest in novel objects and an increase in object directed, repetitive, behavior in the serotonin transporter knockout rats. This behavior was not seen in the SSRI-exposed rats and might be related to the differences in exposure period and the degree of loss of function of the serotonin transporter; the serotonin transporter knockout rats are characterized by a life-long total absence of the serotonin transporter, while in rats perinatally exposed to SSRIs the serotonin transporter knockout is only blocked during the period of exposure. The effects of perinatal SSRI exposure on behavior as described in this chapter are important to take along in the decision about prescribing SSRIs during pregnancy. The fact that most of the behavioral differences we measured were normalized around adolescence and hardly any consequences of perinatal SSRI exposure were observed in adulthood is very positive. Obviously we measured only a small part of the wide range of behaviors an individual can express (e.g. no anxiety and depression-like behavior) and especially the presence of potential long-term effects needs further investigation in the future. In chapter 5 and 6, we investigated changes in genome-wide gene expression in the medial prefrontal cortex (mPFC) in rats with a functionally intact serotonin transporter (wildtype rats) and in serotonin transporter knockout rats. We measured gene expression at 5 different developmental time points, from 8 days after birth (postnatal day (PND) 8) till adulthood. The mPFC is a brain region with a high innervation (supplying a region with nerves) of serotonergic neurons which is involved in, amongst others, the regulation of emotion and its function is reduced in affective disorders. In chapter 5, we showed that gene expression in the mPFC is dynamic across postnatal development and we showed a clear switch around (early-) adolescence from expression of genes involved in neuronal network establishment to genes involved in homeostasis. Homeostasis is the ability of maintaining internal stability regarding chemical and physiological processes in the body. Besides expression of protein-coding genes we also mapped the expression of parts of the DNA which are not coding for proteins itself, but are involved in the regulation of protein-coding genes;

so-called non-coding RNAs. The resource we generated with expression changes across development can be used by other research groups interested in prefrontal cortex development or neurodevelopmental disorders. This resource also provides a good baseline for studying gene expression changes in the mPFC in serotonin-related disorders. As a follow-up to this, we investigated in chapter 6 the gene expression in the mPFC across development in serotonin transporter knockout rats and we compared the expression with the gene expression found in wildtype rats (from chapter 5). We observed most gene expression changes at the earliest time point measured in this study, PND8. At this age we found an enrichment of expression changes in genes involved in neuronal and developmental processes, such as neurotransmission and cell migration. These gene expression changes might cause a dysregulation in neurotransmission and changes in brain development caused by cell migration problems, which might be the underlying cause of the aberrant behavior. In addition, among the genes showing expression changes at PND8 we observed several genes involved in muscle diseases and movement disorders. These genes might play a role in the reduced motor coordination in young serotonin transporter knockout rats observed in chapter 4. More research is needed to confirm this. Furthermore, several genes involved in myelination showed gene expression changes (in general a decrease in expression) in the serotonin transporter knockout rats across development. Our resource with gene expression changes in the serotonin transporter knockout rat can be used for future research into the effects of increased extracellular serotonin levels during development. Finally, we looked into DNA (hydroxy)methylation in the mPFC. The level of DNA (hydroxy)methylation plays a role in the regulation of gene expression and might underlie the observed changes in gene expression. We showed that hydroxymethylation levels increase during postnatal development in the wildtype rats as well as in the serotonin transporter knockout rats. Moreover, we showed an increase in hydroxymethylation at PND35 in de serotonin transporter knockout rats compared to wildtype rats. More research into the meaning of these hydroxymethylation changes is necessary. In chapter 7, we provided an extensive discussion of the research findings summarized above and we proposed directions for future research. The findings in this thesis contribute to an increased insight into the effects of serotonin during different developmental periods. We showed that increased extracellular serotonin levels influence the expression of myelin genes and we showed that the direction of these expression changes is dependent on the time point at which the change in the serotonin system takes place. We showed that both perinatal SSRI exposure and a life-long absence of the serotonin transporter result in a delay in reflex development and development of motor-related behavior. Moreover, we created several resources containing gene expression changes occurring in the hippocampus as a consequence of SSRI treatment in adult rats (chapter 3) and expression changes occuring during mPFC development in wildtype rats (chapter 5) and in serotonin transporter knockout rats (chapter 6). These resources can be used for future research into prefrontal cortex development, neurodevelopmental disorders, the working mechanisms of serotonin and new targets for treatment of affective disorders.

Nederlandse samenvatting

Stemmingsstoornissen zijn psychische aandoeningen waarbij iemands gemoedstoestand dusdanig is verstoord dat diens normale functioneren hierdoor wordt belemmerd. Voorbeelden van dergelijke stoornissen zijn depressie, angststoornissen en bipolaire stoornissen. Stemmingsstoornissen zijn veel voorkomende ziektes in de hersenen waarvan de oorzaak ligt in een combinatie van genetische factoren en omgevingsinvloeden. Een belangrijk molecuul in de hersenen dat betrokken is bij het ontstaan van stemmingsstoornissen is serotonine. Serotonine (ook wel genoemd 5-hydroxytryptamine, 5-HT) is een neurotransmitter (een boodschapper die zorgt voor informatieoverdracht tussen zenuwcellen) geproduceerd in serotonerge neuronen, die een belangrijke rol speelt bij processen als stemming, slaap, seksuele activiteit, emotie en eetlust. Serotonine wordt via blaasjes vrijgelaten in de extracellulaire ruimte en kan dan binden aan serotonine receptoren op andere neuronen om zo verschillende processen te activeren. Serotonine transporters (5-hydroxytryptamine transporter, 5-HTT) gelegen op de serotonerge neuronen kunnen serotonine heropnemen in het neuron en hiermee de hoeveelheid beschikbare, extracellulaire serotonine regelen. Decennia geleden is er aangetoond dat een verlaagd extracellulair serotonine niveau in de hersenen geassocieerd is met stemmingsstoornissen zoals depressie. Daarnaast is ook aangetoond dat genetische variatie (zoals polymorfismen; natuurlijke variaties in de DNA sequentie die met relatief hoge frequentie voorkomen in de populatie) in serotonine genen geassocieerd is met stemmingsstoornissen. Een veel bestudeerd polymorfisme is het 5-HTTLPR (5-HTT-linked polymorphic region) polymorfisme gelegen in het stukje DNA dat de expressie van de serotonine transporter regelt. De korte variant van dit polymorfisme (S (short) allel; bevat minder herhalingen van een repeterende stukje DNA) is geassocieerd met lagere niveaus van de serotonine transporter en mensen met deze variant hebben een verhoogde kans op depressie en angststoornissen.

Het serotonine systeem is een erg belangrijk aangrijpingspunt voor veel medicijnen die voorgeschreven worden bij stemmingsstoornissen. De medicijnen die het meest worden voorgeschreven voor stemmingsstoornissen zijn selectieve serotonine heropname remmers (selective serotonin reuptake inhibitors, SSRIs). SSRIs blokkeren de serotonine transporter en daarmee de heropname van serotonine in het neuron, wat resulteert in hogere extracellulaire serotonine niveaus. SSRIs zijn vooral bekend als antidepressiva, maar ze worden daarnaast ook voorgeschreven voor o.a. angststoornissen. Ondanks de vele positieve effecten hebben klinische studies aangetoond dat SSRIs ook verschillende bijwerkingen kunnen hebben, zoals misselijkheid, verminderde eetlust, agressie/geweld, verstoorde rapid eye movement (REM)-slaap en stoornissen op seksueel gebied. Een ander nadeel is dat het vaak weken duurt voordat de klinische effecten van SSRIs merkbaar zijn.

Depressie komt relatief vaak voor tijdens de zwangerschap en SSRIs worden in deze periode veel voorgeschreven. Studies hebben aangetoond dat SSRIs via de placenta het ongeboren kind kunnen bereiken en na de geboorte via de moedermelk kunnen worden overgebracht. Er is aangetoond dat deze vroege blootstelling aan SSRIs nadelige effecten

kan hebben op de ontwikkeling van het kind, zoals verhoogde kans op vroeggeboorte, laag geboortegewicht, serotonerge onttrekkingsverschijnselen van de pasgeborene, defecten in motoriek, autistisch gedrag, verhoogde bloeddruk in de longen (pulmonaire hypertensie) en aangeboren hartafwijkingen. Daarnaast is met name in dierstudies aangetoond dat SSRI blootstelling rond de geboorte een verhoogde kans op stemmingsstoornissen op volwassen leeftijd met zich meebrengt. SSRI blootstelling op jonge leeftijd verhoogd dus de kans op angst en depressieve symptomen op volwassen leeftijd, terwijl SSRI behandeling op volwassen leeftijd deze symptomen juist onderdrukt. Belangrijk is om hierbij te vermelden dat onbehandelde stemmingsstoornissen van de moeder ook nadelige effecten kunnen hebben op het (ongeboren) kind. Samenvattend, SSRIs laten leeftijdsafhankelijke effecten zien met negatieve gevolgen bij blootstelling op jonge leeftijd. Interessant daarbij is het tegengestelde leeftijdsafhankelijke effect op het gebied van angst en depressie. Daarnaast zien we bij gebruik op volwassen leeftijd nog relatief veel bijwerkingen. Dit alles geeft aan dat er meer kennis over de oorzaak van stemmingsstoornissen en de effecten van serotonine en SSRIs op zowel volwassen leeftijd als tijdens de ontwikkeling nodig is om de behandeling van stemmingsstoornissen te verbeteren.

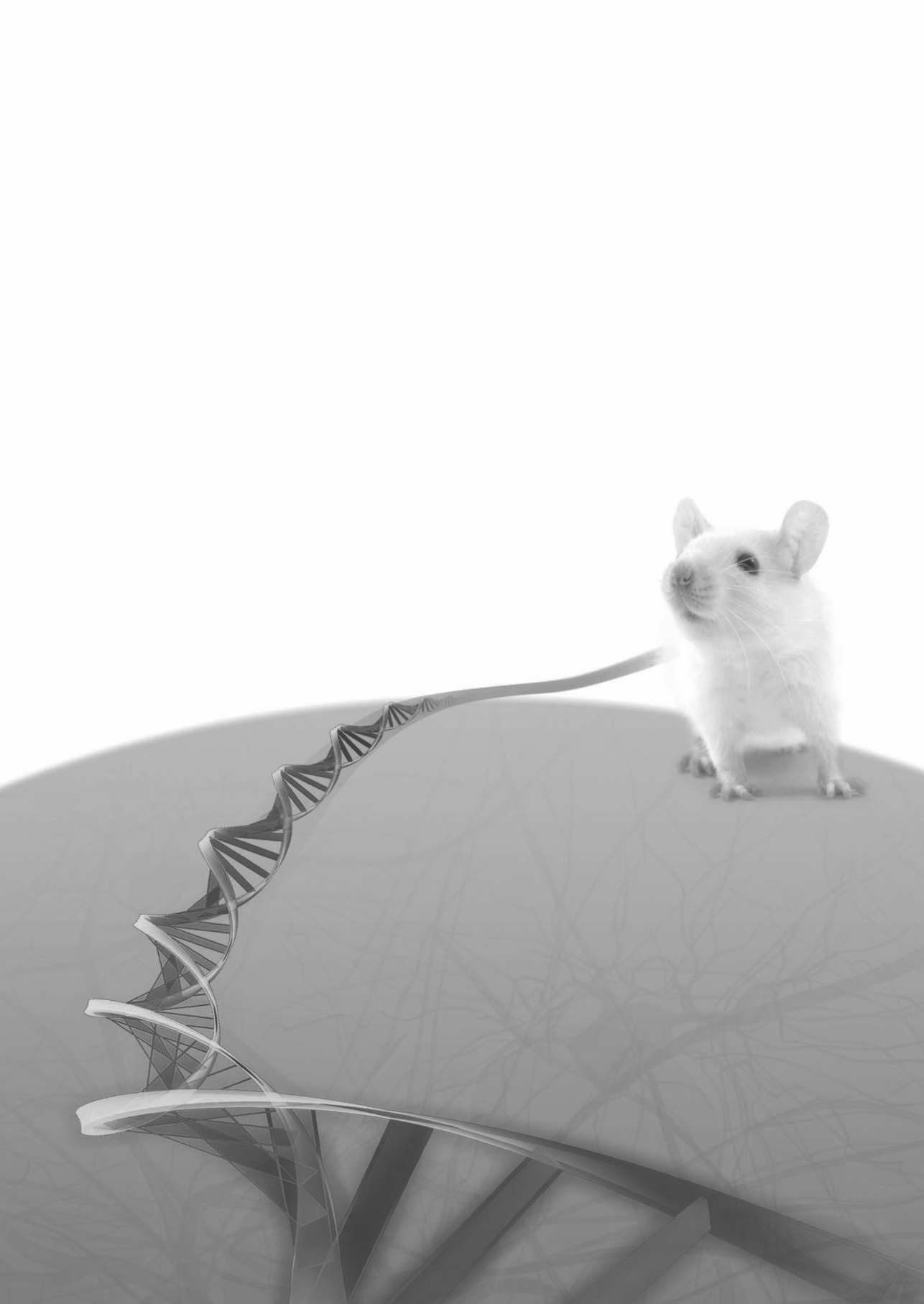
In dit proefschrift hebben we ons gericht op de moleculaire veranderingen die plaatsvinden in de hersenen als gevolg van het verhogen van de extracellulaire serotonine niveaus, zowel tijdens de postnatale (na de geboorte) ontwikkeling als op volwassen leeftijd. Daarnaast hebben we gekeken naar effecten van het verhogen van de extracellulaire serotonine niveaus op jonge leeftijd op het gedrag vanaf vlak na de geboorte tot aan volwassen leeftijd.

In hoofdstuk 2 hebben we een samenvatting gegeven van de effecten van SSRI behandeling op genexpressie in de hersenen die beschreven waren in de beschikbare literatuur aan het begin van mijn promotietraject. De meerderheid van de genen waarvan de expressie beïnvloed wordt door SSRIs zijn genen die coderen voor de serotonine receptoren, componenten van niet-serotonerge neurotransmitter systemen, neurotrofe factoren (factoren die een bevorderende werking hebben op celdeling, ontwikkeling en overleving), hormonen van de hypothalamus en ontstekingsfactoren. Verder viel op dat de meeste studies gekeken hebben naar genexpressie 24 uur na de laatste SSRI behandeling, terwijl onderzoek naar de lange termijneffecten waardevolle aanvullende informatie kan opleveren over de werkingsmechanismen van SSRIs. Daarnaast is er nauwelijks onderzoek gedaan naar de effecten van SSRI blootstelling rond de geboorte op genexpressie. In hoofdstuk 3 hebben we dan ook gekeken naar de lange termijneffecten van SSRI behandeling op genexpressie in de hippocampus, zowel na SSRI blootstelling op volwassen leeftijd als na SSRI blootstelling rond de geboorte. De hippocampus is een hersengebied dat is aangetast bij stemmingsstoornissen en voornamelijk betrokken is bij het opslaan van herinneringen. Wij hebben in volwassen ratten aangetoond dat de expressie van verschillende genen betrokken bij myelinatie verhoogd is in de hippocampus 40 dagen na de laatste SSRI behandeling in vergelijking met een controle groep die geen SSRIs kreeg. Myelinatie is het vormen van myeline rondom

axonen wat ervoor zorgt dat signalen sneller worden doorgegeven. Verder hebben we laten zien dat deze genexpressie niveaus in deze ratten negatief correleren met de mate van angst getest m.b.v. een gedragstest; de hogere expressie van deze myelinatie genen correleerde met een lager angstniveau. Verder onderzoek zou moeten uitwijzen of deze verhoogde genexpressie weer omlaag gaat bij een terugkeer van de depressieve klachten en of myelinatie een mogelijke diagnostische marker zou kunnen zijn voor het voorspellen van de kans op een terugval. Verder hebben we in dit hoofdstuk aangetoond dat bij SSRI blootstelling vlak na de geboorte de expressie van genen betrokken bij myelinatie juist verlaagd is in de hippocampus als deze dieren volwassen zijn en dat deze expressie ook negatief correleert met de mate van angst; de lagere expressie van de myelinatie genen correleerde met een hoger angstniveau. Samengevat zien we een verhoogde expressie van myelinatie genen na volwassen blootstelling en een verlaagde genexpressie naar blootstelling rond de geboorte. Dit tegengestelde leeftijdsafhankelijke effect van SSRIs op expressie niveaus van myelinatie genen past goed bij de tegengestelde effecten op angst en depressief gedrag die al veelvuldig in de literatuur beschreven zijn. In hoofdstuk 4 hebben we onderzocht wat de effecten van veranderingen in het serotonine systeem, namelijk verhoogde serotonine niveaus, zijn op de (gedrags) ontwikkeling. Er zijn al enkele vroege gedragseffecten van SSRI blootstelling op jonge leeftijd beschreven in de literatuur, maar de effecten op de verdere ontwikkeling van de dieren waren nog onbekend. Daarnaast was er nog geen onderzoek gedaan naar de effecten van het hebben van de korte variant (S-allel) van het serotonine transporter polymorfisme op de (gedrags)ontwikkeling. We hebben dit verder onderzocht in diermodellen en zagen dat zowel ratten die perinataal (rond de geboorte) waren blootgesteld aan SSRIs, als ratten die hun hele leven lang de serotonine transporter misten (zogenaamde serotonine transporter knockout ratten, een model voor 5-HTTLPR S-allel dragers), een vertraging in ontwikkeling vertonen, met name op het gebied van motorcoördinatie en reflexontwikkeling. Met uitzondering van verminderd voortbewegen (hypolocomotion), waren rond adolescentie de meeste verschillen in de ontwikkeling weer verdwenen. Op adolescente en volwassen leeftijd zien we in de serotonine transporter knockout ratten verminderde interesse in nieuwe objecten en meer object-gericht, repetitief, gedrag. Dit gedrag werd echter niet waargenomen in de ratten blootgesteld aan SSRIs en zou gerelateerd kunnen zijn aan de verschillen in de periode en de mate van functieverlies van de serotonine transporter; de serotonine transporter knockout ratten worden gekenmerkt door levenslange totale afwezigheid van de serotonine transporter en in de ratten die perinataal zijn blootgesteld aan SSRIs is de serotonine transporter alleen tijdens blootstelling geblokkeerd. De in dit hoofdstuk gevonden gedragseffecten zijn belangrijk om mee te nemen in de beslissing over het al dan niet voorschrijven van SSRIs tijdens de zwangerschap. Het feit dat de meeste door ons gemeten verschillen in gedrag weer zijn verdwenen rond adolescente leeftijd en er op volwassen leeftijd nauwelijks gevolgen te zien zijn van SSRI blootstelling is positief. Wij hebben uiteraard maar een klein deel van het gedrag bestudeerd (o.a. geen angst en depressief gedrag) en met name de aanwezigheid van eventuele lange termijneffecten van perinatale SSRI blootstelling moet in de toekomst nog

uitgebreider worden onderzocht. In hoofdstuk 5 en 6 hebben we gekeken naar veranderingen in genoom-brede genexpressie in de mediale prefrontale cortex (mPFC) in ratten met een intacte serotonine transporter expressie (wildtype ratten) en in serotonine transporter knock-out ratten. Genexpressie hebben we gemeten op 5 verschillende tijdspunten tijdens de ontwikkeling, vanaf dag 8 na de geboorte tot volwassen leeftijd. De mPFC is een hersengebied met een hoge innervatie (voorziening van het gebied met zenuwen) van serotonerge neuronen dat betrokken is bij onder andere emotieregulatie en minder goed lijkt te functioneren bij verschillende stemmingsstoornissen. In hoofdstuk 5 hebben we laten zien dat genexpressie dynamisch is tijdens de postnatale ontwikkeling van de hersenen en dat er een duidelijke omschakeling plaatsvindt rond (jong-)adolescente leeftijd van expressie van genen betrokken bij het vormen van neuronale netwerken, naar genen betrokken bij homeostase. Homeostase is het in evenwicht houden van de chemische en fysiologische processen in het lichaam. Naast genen die daadwerkelijk coderen voor eiwitten hebben we ook de expressie in kaart gebracht van stukjes erfelijk materiaal die zelf niet coderen voor eiwitten, maar betrokken zijn bij de regulatie van eiwit-coderende genen; de zogenaamde niet-coderende RNAs. We hebben een dataset gecreëerd met expressieveranderingen tijdens de ontwikkeling welke gebruikt kan worden door andere onderzoekers die geïnteresseerd zijn in prefrontale cortex ontwikkeling of neuronale ontwikkelingsstoornissen. Daarmee vormt hoofdstuk 5 een goede basis voor het bestuderen van veranderingen in genexpressie in de mPFC bij serotonine gerelateerde ziektes. Als vervolg hierop hebben we in hoofdstuk 6 gekeken naar expressie in de mPFC tijdens de ontwikkeling in serotonine transporter knock-out ratten en deze expressie hebben we vergeleken met de genexpressie in ratten met een intacte serotonine transporter (uit hoofdstuk 5). We zagen de meeste genexpressie veranderingen op de jongste leeftijd die we gemeten hadden, 8 dagen na de geboorte. Op die leeftijd vonden we een verrijking aan expressie veranderingen (zowel verhoogde als verlaagde expressie) in genen betrokken bij neuronale en ontwikkelingsprocessen zoals neurotransmissie en celmigratie. Deze veranderingen in genexpressie zouden kunnen zorgen voor een afwijkende signaaloverdracht tussen zenuwen (neurotransmissie) en afwijkingen in de hersenontwikkeling door problemen met celmigratie, die mogelijk de onderliggende oorzaak zijn van het afwijkend gedrag. Onder de genen die expressieveranderingen lieten zien 8 dagen na de geboorte zagen we ook veel genen die betrokken zijn bij spierziekten en bewegingsstoornissen. Deze genen zouden een rol kunnen spelen bij de in hoofdstuk 4 beschreven verminderde motorcoördinatie in jonge serotonine transporter knockout ratten. Om dit te bevestigen is meer onderzoek nodig. Daarnaast bleken enkele genen betrokken bij myelinatie veranderingen in genexpressie te laten zien (over het algemeen verlaagde expressie) in de serotonine transporter knockout ratten gedurende de gehele ontwikkeling. Onze dataset met veranderingen van genexpressie in de serotonine transporter knockout ratten kan gebruikt worden voor verder onderzoek naar de effecten van verhoogde extracellulaire serotonine niveaus tijdens de ontwikkeling. Tot slot hebben we gekeken naar DNA (hydroxy)methylatie in de mPFC. De mate van DNA (hydroxy)methylatie speelt een rol bij

de regulatie van genexpressie en zou daarmee ten grondslag kunnen liggen aan de waargenomen veranderingen in genexpressie. We hebben aangetoond dat het hydroxymethylatie niveau toeneemt gedurende de postnatale ontwikkeling zowel in de wildtype als in de serotonine transporter knockout ratten. Daarnaast zagen we een toename in hydroxymethylatie in 35 dagen oude serotonine transporter knockout ratten in vergelijking met de wildtype ratten. Verder onderzoek is nodig naar de betekenis van deze veranderingen in hydroxymethylatie niveaus. In hoofdstuk 7 hebben we onze resultaten uitgebreid bediscussieerd en daarnaast hebben we vervolgexperimenten voorgesteld. De bevindingen in dit proefschrift hebben een belangrijke bijdrage geleverd aan het verkrijgen van meer inzicht in de effecten van serotonine tijdens de verschillende fasen van de ontwikkeling. We hebben aangetoond dat verhoogde extracellulaire serotonine niveaus effect hebben op de expressie van myelinatie genen en dat de richting van dit effect afhankelijk is van de leeftijd waarop de verandering in het serotonine systeem plaatsvindt. We hebben laten zien dat zowel SSRI blootstelling op jonge leeftijd als het levenslang uitschakelen van de serotonine transporter zorgt voor een tijdelijke achterstand in ontwikkeling van motorcoördinatie en reflexen. We hebben daarnaast verschillende genexpressie datasets gecreëerd met expressieveranderingen die optreden in de hippocampus als gevolg van SSRI behandeling in volwassen ratten (hoofdstuk 3) en expressieveranderingen die optreden tijdens mPFC ontwikkeling in wildtype ratten (hoofdstuk 5) en in serotonine transporter knockout ratten (hoofdstuk 6) . Deze datasets kunnen gebruikt worden voor verder onderzoek naar prefrontale cortex ontwikkeling, neuronale ontwikkelingsziekten, de werkingsmechanismen van serotonine en nieuwe aangrijpingspunten voor medicatie voor stemmingsstoornissen.



9

Dankwoord
Curriculum Vitae
List of publications
List of abbreviations
Donders Series

Dankwoord

Het is zover, mijn proefschrift is klaar! Een mooi resultaat van een onvergetelijke tijd. Dit werk had ik niet kunnen doen zonder de steun van vele mensen om mij heen.

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Yvet

Curriculum Vitae

Yvet Kroeze werd geboren op 19 oktober 1985 te Almelo. Ze groeide op in Borne en behaalde in 2004 haar VWO-diploma aan Scholengemeenschap De Grundel te Hengelo. Hierna begon ze aan haar Bachelor Biologie en Medisch laboratorium onderzoek aan de Saxion Hogeschool te Enschede, welke werd afgerond in augustus 2007 (*Cum laude*). Hierna begon ze aan de Master Medische Biologie aan de Radboud Universiteit Nijmegen. Tijdens haar Master programma heeft ze twee wetenschappelijke stages gedaan. Haar eerste wetenschappelijke stage heeft ze gelopen bij de afdeling Nierziekten van het Radboudumc. Onder begeleiding van Dr. Casandra van Bavel en Dr. Johan van der Vlag heeft ze onderzoek uitgevoerd naar histon modificaties bij de auto-immuunziekte systemische lupus erythematosus. Tijdens haar tweede Masterstage, bij de afdeling Genetica op het Radboudumc, heeft ze onderzoek gedaan naar de betrokkenheid van het gen *BTG1* bij het ontstaan van acute lymfatische leukemie bij kinderen onder begeleiding van Dr. Esme Waanders en Dr. Roland Kuiper. Na het behalen van haar Master diploma in 2010 (*bene meritum*) is ze begin 2011 begonnen aan haar promotieonderzoek binnen de afdeling Cognitieve Neuroscience van het Donders institute for Brain, Cognition and Behaviour en de afdeling Genetica van het Radboudumc onder leiding van Judith Homberg, Huiqing Zhou en Hans van Bokhoven. Tijdens dit promotietraject heeft ze onderzoek gedaan naar de effecten van het serotonine systeem op gedrag en gen expressie. De resultaten van het onderzoek zijn beschreven in dit proefschrift en daarnaast zijn de bevindingen gepresenteerd op verschillende (inter)nationale congressen, zoals de Society for Neuroscience, Donders Discussions, Dutch Neuroscience meeting en het symposium van de Nederlandse Vereniging voor Farmacologie. Tijdens haar promotieonderzoek heeft ze verschillende cursussen gevolgd waaronder Statistiek voor promovendi en Writing course “On papers and publishing”. Na haar promotieonderzoek is ze gaan werken bij de afdeling Kindergeneeskunde waar ze zich bezig houdt met datamanagement van klinische trials in met name de kinderoncologie.

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Kroeze Y*, Oti M*, Cooijmans R, Beusekom E, Kroeze LI, Middelman A, van Bokhoven H, Kolk SM, Homberg JR#, Zhou H#. Genetic inactivation of the serotonin transporter dysregulates expression of neurotransmission and myelination genes in the postnatal medial prefrontal cortex of the rat. 2017. *In preparation*

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List of abbreviations

Abbreviations of gene names, protein names and domain names are not included in the list

5-HT	5-hydroxytryptamine; serotonin
5-HTT transporter	5-hydroxytryptamine transporter; serotonin
5-HTTLPR	5-hydroxytryptamine transporter-linked polymorphic region
5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
AC	Adenylyl cyclase
ACC	Anterior cingulate cortex
ACTH	Adrenocorticotropic hormone
AD(H)D	Attention deficit (hyperactivity) disorder
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ANOVA	Analysis of variance
ASD	Autism spectrum disorder
Avp	Arginine vasopressin
BDNF	Brain-derived neurotrophic factor
CA	Cornu Ammonis
cDNA	Complementary deoxyribonucleic acid
ChIP-seq	Chromatin immuno-precipitation followed by sequencing
CMS	Chronic mild stress
CNS	Central nervous system
COMT	Catechol-O-methyltransferase
CpG	Cytosine-phosphate-guanine
CREB	cAMP-response element-binding protein
CRF	Corticotrophin releasing factor
CSF	Cerebrospinal fluid
D2	Discrimination index
DA	Dopamine
DAVID	Database for Annotation, Visualization and Integrated Discovery
DG	Dentate gyrus
DLPFC	Dorsolateral prefrontal cortex
DNMT	DNA methyltransferases
DRN	Dorsal raphe nucleus
DSM-5	Diagnostic and Statistical Manual of Mental Disorders, fifth revision
E(2)	Estradiol
ES cell	Embryonic stem cell
FACS	Fluorescence-activated cell sorting
FPKM	Fragments Per Kilobase per Million mapped reads
GABA	Gamma-aminobutyric acid
Gap-43	Growth-associated protein-43
GD	Gestational day
GO	Gene ontology
GR	Glucocorticoid receptor
GSK3B	Glycogen synthase kinase-3beta
GWAS	Genome-wide association studies
HDAC	Histone deacetylase
HPA-axis	Hypothalamus-pituitary-adrenal axis
HTR	Hydroxytryptamine receptor
ID	Intellectual disability
IDO	Indoleamine 2,3-dioxygenase
LCLs	Lymphoblastoid cell lines
lincRNA	Long intergenic non-coding RNA
MAOI	Monoamine oxidase inhibitor
MDD	Major depressive disorder
miRNA	micro ribonucleic acid
mPFC	Medial prefrontal cortex
MR	Mineralocorticoid receptor
mRNA	messenger ribonucleic acid
mTOR	Mechanistic target of rapamycin
ncRNA	non-coding ribonucleic acid
NE	Norepinephrine
NMDA	N-Methyl-D-aspartic acid
NOR	novel object recognition
NPY	Neuropeptide Y
NRI	Norepinephrine reuptake inhibitor
NSFT	Novelty-suppressed feeding test
OCD	Obsessive compulsive disorder
ODB	Object directed behavior
OFC	Orbitofrontal cortex
OFCo	Time spent in the corner
OFCe	Time spent in the center
OFT	Open field test
OPC	Oligodendrocyte progenitor cell
Oxt	Oxytocin
PC	Principal component
PCA	principal component analysis
PCC	Pearson correlation coefficient
PCR	Polymerase chain reaction
PDE	cAMP-specific phosphodiesterases
PFC	Prefrontal cortex
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PND	Postnatal day
POMC	Proopiomelanocortin
PPHN	Persistent pulmonary hypertension
PPI	Prepulse inhibition
PTSD	Post-traumatic stress disorder
PVN	Paraventricular nucleus
qPCR	Quantitative polymerase chain reaction
REM	Rapid eye movement
RNA	Ribonucleic acid
RNA-seq	Ribonucleic acid sequencing
RT	Reverse transcription
SCZ	Schizophrenia
S.E.M	Standard error of the mean
SGZ	Subgranular zone
siRNA	Small interfering ribonucleic acid
SNP	Single nucleotide polymorphism
SNRI	Serotonin-norepinephrine reuptake inhibitor
SPSS	Statistical Package for the Social Sciences
SSRIs	Selective serotonin reuptake inhibitors
TCA	Tricyclic antidepressant
TCAs	Thalamocortical axons
tPA	Plasminogen activator
Tph	L-tryptophan hydroxylase
VEGF	Vascular endothelial growth factor
VLPFC	Ventrolateral prefrontal cortex
VZ	Ventricular zone

Donders Graduate School for Cognitive Neuroscience

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